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TRANSCRIPTIONAL REGULATION OF MOUSE RIBOSOMAL RNA SYNTHESIS BY  
PATHWAYS INVOLVING PROTEIN KINASE C, CALCIUM, INSULIN, AND SERUM



Mark Lyndon Grotke



# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of the collection of information, including suggestions for reducing this burden, to Washington Headquarters Service, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 3 May 1992		3. REPORT TYPE AND DATES COVERED Ph.D Dissertation	
4. TITLE AND SUBTITLE Transcriptional Regulation of Mouse Ribosomal RNA Synthesis by Pathways Involving Protein Kinase C, Calcium, Insulin, and Serum.				5. FUNDING NUMBERS AR621-1 Fully funded civil schooling	
6. AUTHOR(S) MAJ Mark L. Grotke					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) US Army Soldier Support Center Ft. Benjamin Harrison, IN 46216  AV 699-5649 Comm. 317-549-5649				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) US Army Military Personnel Center ATTN: DAPC-OPB-D 200 Stovall Street Alexandria, VA 22332-0400				10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Ph.D. dissertation prepared at the University of Southern California					
12a. DISTRIBUTION/AVAILABILITY STATEMENT  <div style="border: 1px solid black; padding: 5px; width: fit-content; margin: 10px auto;">This document has been approved for public release and sale; its distribution is unlimited.</div>				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) We probed individual regions of the rDNA gene of Mouse Macrophage J774. A1 tissue culture cells for transcriptional activity by using nuclear run-on assays. We determined that the intergenic spacer region (IGS), the external transcribed sequences (ETS), and the 28S regions of the rDNA gene are transcribed and apparently under regulatory control, both in cells that are rapidly growing in 10% Fetal bovine serum supplemented media and in slower growing cells in 2% fetal bovine supplemented media. The IGS region of the rDNA is transcribed at approximately 20% of the rate of the ETS region. The ENH region of the rDNA gene is transcribed at a low rate, which did not change when cells were stimulated with TPA A23187, insulin, or serum. The ETS region was transcribed at approximately 20% of the rate of the 28S region. These observations suggest processing of the rDNA transcripts from the ETS region occur rapidly in intact cells, or that the structural regions of the gene (18S, 5.8S, and 28S) are transcribed at a higher rate than the ETS region. We demonstrate that transcription occurs upstream of the spacer promoter.					
14. SUBJECT TERMS  Ribosomal RNA, Transcription, Macrophage Cells rRNA transcriptional regulation				15. NUMBER OF PAGES 111	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT  UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE  UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT  UNCLASSIFIED	20. LIMITATION OF ABSTRACT		

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by

Mark Lyndon Grotke

A Dissertation Presented to the  
FACULTY OF THE GRADUATE SCHOOL  
UNIVERSITY OF SOUTHERN CALIFORNIA  
In Partial Fulfillment of the  
Requirements for the Degree  
DOCTOR OF PHILOSOPHY  
(Molecular Biology)

May 1992

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Capt Jim Creighton TAPC/OPB-D  
Alexandria, VA 22332-0411

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## DEDICATION

To my dear wife, Colleen, who has followed me in my wanderings all over the world without complaint. Without her I would be much less. Also to my children, Matthew and Caitlin. Now Daddy will be home while it is "still light time."

## ACKNOWLEDGMENTS

I want to acknowledge the people and institutions that have made my graduate career possible. Many thanks to my advisor, Dr. Heather Weber. She not only kept me on the straight and narrow, but she has become a close friend as well. One can't ask for anything more.

Also, thanks to Drs. Maria Pellegrini, Arnold Dunn, M. Michael Appleman, and Gibson Reaves. Their guidance, comments, help, and suggestions have been enormously beneficial.

Finally, I want to salute the men and women of the United States Army, and especially Army Aviation. Without them I would not be here. "ABOVE THE BEST."

April, 1992    Los Angeles, California

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## LIST OF ABBREVIATIONS

A <sub>260</sub>	absorbance at 260 nm
A <sub>280</sub>	absorbance at 280 nm
bp	base pair
cpm	counts per minute
DEP	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis-( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid
ENH	enhancer region
ETS	external transcribed sequence
FBS	fetal bovine serum
hepes	N-2-hydroxyethylpiperazine-N'2- ethanesulfonic acid
IGS	intergenic spacer
kb	kilo base
°C	degrees Celsius
PMSF	phenylmethylsulfonyl fluoride
pol I	RNA polymerase I
pol II	RNA polymerase II
pol III	RNA polymerase III

rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
RNase A	ribonuclease A
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate buffer
TAE	tris/acetate buffer
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris hydrochloride
TPA	12-0-tetradecanoylphorbol-13-acetate
UTP	uridine 5'-triphosphate
uv	ultraviolet
xg	relative centrifugal force

## ABSTRACT

We are interested in understanding the pathways that regulate transcription of rRNA. We probed individual regions of the rRNA gene of mouse macrophage J774.A1 tissue culture cells for transcriptional activity by using nuclear run-on reactions conducted in the presence of alpha-amanitin. We determined that the intergenic spacer region (IGS), the external transcribed sequences (ETS), and the 28S regions of the rRNA gene are actively transcribed and apparently under regulatory control. The IGS region of the rDNA is transcribed at low rate, and is responsive to stimulation by TPA, A23187, insulin, and serum. The ENH region of the rRNA gene is transcribed at a low rate, which did not change when cells were stimulated with TPA, A23187, insulin, or serum. The ETS region was transcribed at about one-fifth the rate of the 28S region. These observations suggest that rRNA transcription is regulated by the cell, and that processing of rRNA transcripts from the ETS region occur rapidly in intact cells, or that the structural regions of the gene (18S, 5.8S, and 28S) are transcribed at a higher rate than the ETS region.

We also demonstrate that transcription is occurring in the IGS upstream of the spacer promoter, but that no transcription is occurring between the  $T_0$  terminator and the +1 start site. These observations have implications for the *in vivo* function of the spacer promoter and the  $T_0$  terminator.

Additionally, by detecting a stimulatory rRNA transcriptional response after treating J774.A1 cells with TPA, A23187, insulin, we have implicated the insulin receptor as well as protein kinase C, and calcium in regulatory control pathways of rRNA transcription. Rapidly growing J774.A1 cells produced larger stimulatory responses to treatment, than did slowly growing cells, suggesting that the implicated pathways may be influenced by the growth state of the cell. Serum stimulation produced the largest increase in rRNA transcription in rapidly growing cells, while TPA, A23187, and insulin produced smaller increases. These observations suggest that multiple pathways may regulate rRNA transcription in mouse macrophage cells.

## INTRODUCTION

Ribosomal RNA (rRNA) accounts for about 50-80% of a cell's total RNA (Sollner-Webb and Mougey, 1991; Sollner-Webb and Tower, 1986 ). In the 1960's and 1970's the dominant view was that this large pool of rRNA was relatively static and unperturbed by transient changes in the cell's physiological state. Indeed, this view persists to some degree today, but in the past decade this view of a static rRNA pool has been challenged. While it seems clear that one will not observe the thousand fold changes that can be observed under certain conditions with transcription of messenger RNA genes (for example see Suva *et al.*, 1991 Dean *et al.*, 1989), it appears that rRNA transcription does respond rapidly to transient changes in the cell's physiological state.

### Structure and function of the ribosomal RNA gene elements.

Any study of rDNA transcription regulation must take into account the structure and function of the elements that compose the rRNA genes. In general, the rRNA genes are arranged as a series of tandem repeats oriented head to tail. *Mus musculus* has about 100 haploid copies of the rRNA gene (Gaubatz and Cutler, 1978) which are divided between chromosomes 12, 16, and 18 (Elsevier and Ruddell, 1975). Other workers have located the rRNA gene repeats on chromosomes 15, 18, and 19 in inbred strains of mice (Henderson *et al.*, 1974).

Not all of the approximately 100 haploid copies of the rRNA genes are actively transcribed in a cell at any given time (Sollner-Webb and Tower, 1986). During early developmental stages, when growth is rapid, many or most of the rRNA genes are active. At other times a smaller number of the rRNA genes are actively being transcribed, with the rest being sequestered into nucleosomes which are transcriptionally inactive (Conconi *et al.*, 1989).

The general structure of a rRNA gene is shown in Figure 1. Each rRNA gene repeat is about 44 kb long (Grummt and Gross, 1980; Kominami *et al.*, 1980; Arnhiem, 1979; Cory and Adams, 1977). For simplicity we have the

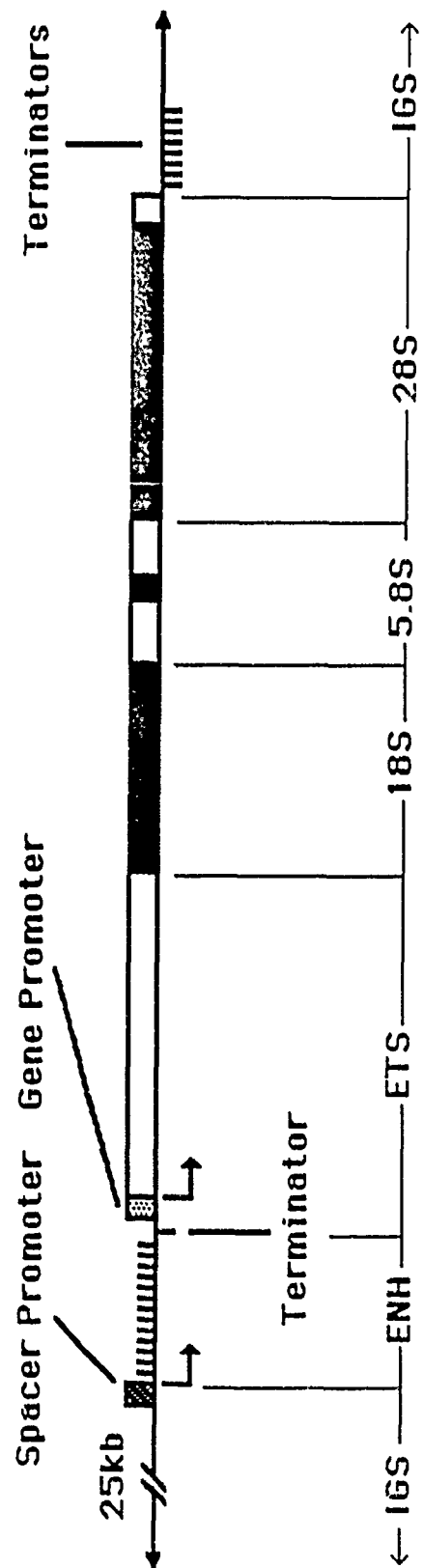
Figure 1. The structure of Mouse ribosomal DNA.

Mouse rDNA genes are arranged in tandem arrays, with each gene region encoding the 18S, 5.8S, and 28S structural rRNAs. The rDNA is divided into four regions for the purposes of this study. The genes are separated by a largely uncharacterized 25 kb region termed the Intergenic Spacer (IGS) region. The spacer promoter, a region of homology with the gene promoter, is located at the 3' terminus of the IGS region. The Enhancer (ENH) region contains thirteen 135 bp repeated elements. The exact mode of enhancement of rRNA transcription is unclear, however, the enhancer repeats have been shown to bind polymerase I transcription factors. The 3' end of the ENH is bounded by the  $T_0$  terminator. This region has been shown to bind a factor which is sufficient to terminate a transcribing polymerase I molecule. Immediately downstream of the  $T_0$  terminator is the gene promoter region, which is composed of an upstream element and a downstream element. Transcription begins at the +1 site, and continues downstream through the External Transcribed Segment (ETS). The ETS is a leader RNA which is rapidly processed off of the structural RNA molecules and degraded. The 18S, 5.8S, and 28S rRNAs, when processed out of the pre-ribosomal transcript, will be incorporated into ribosomal units. Downstream of the 3' end of the 28S rRNA is a series of eight repeated terminators, termed  $T_1$ - $T_8$ . These elements terminate transcription and cause the elongating polymerase I molecules to release their nascent transcripts. Arrows at the spacer promoter and the gene promoter indicate transcription start sites. Filled boxes indicate structural genes.



Figure 1

Mouse Ribosomal DNA



rRNA gene set divided into regions: the Intergenic Spacer region (IGS), the Enhancer Region (ENH), the External Transcribed Spacer region (ETS) and the 18S, 5.8S and 28S coding regions.

The IGS is the least understood region of the rRNA gene. It is also the largest, accounting for about 25kb of the gene's length. Only a small portion of the 5' and 3' ends of the IGS have been sequenced and characterized (Kuhn and Grummt, 1987; Grummt *et al.*, 1986). At the 5' end of the IGS (as viewed from the +1 transcription start site) is a series of eight repeated elements, termed T<sub>1</sub>-T<sub>8</sub>, that are each 18 bp long, with the consensus sequence AGGTCGACCAG(A/T)(T/A)NTCCG. This consensus sequence carries a Sal I restriction site, and is termed a "Sal box". This sequence motif, together with a bound protein factor (TTF-1, Kuhn *et al.*, 1988) is sufficient to direct termination of the rRNA polymerase (pol I) and to cause the release of the elongating transcript. (Kuhn and Grummt, 1989; Kuhn *et al.*, 1988; Bartsch *et al.*, 1988; Grummt *et al.*, 1986; Kominami *et al.*, 1982). At the 3' end of the IGS is a structure that has been termed the "spacer promoter" because of the sequence similarity that it bears with the gene promoter. Comparing the spacer promoter to the core promoter:

	-1978	-1960
spacer promoter	TCGTACAGGGAGATGGCCA	
core promoter	<u>ITGGAC</u> CT <u>GGAGATA</u> <u>GCTA</u>	
	-17	+1

one can see that 12 of the 18 positions are conserved (conserved bases are underlined; Kuhn and Grummt, 1987). Spacer promoters have been found in most species examined, including *Drosophila*, *Xenopus*, Chinese hamster, rat, human and probably yeast and wheat (Reeder, 1989 and references therein; Reeder, 1984). The spacer promoters have been shown to initiate transcription both *in vitro* and *in vivo* (Kuhn and Grummt, 1987; Michelson and Moss, 1987; DeWinter and Moss, 1986; Moss, 1983). The actual function of the spacer promoter is, however, not clear. Moss has proposed that the spacer promoter functions as a transcription machinery magnet, in that it attracts transcriptional machinery to the spacer promoter and then hands these factors off to the gene promoter by a method termed "readthrough enhancement". In Moss's view, the polymerase and associated factors are either pushed through the region between the spacer promoter and the gene promoter by other polymerases, or translocate

through this region without producing a rRNA transcript. Once through this intervening region, the polymerase would terminate at the promoter proximal terminator,  $T_0$ , release its nascent transcript (if any), and then be available for use at the gene promoter. Moss was led to postulate that the polymerase moved or was pushed through the region between the spacer promoter and the gene promoter without producing a transcript because of the paucity of transcripts from this region. Some workers have reported they cannot detect transcription (Wood *et al.*, 1984), while others have reported low levels of transcription originating from the spacer promoter (Harrington and Chickaraishi, 1987; Kuhn and Grummt, 1987). It now seems clear that *in vivo* there is a low level of transcription occurring in this region (Sollner-Webb and Mougey, 1991; Reeder, 1989; Morgan *et al.*, 1984).

Reeder, on the other hand, has proposed that the spacer promoter initiates a low level of transcription in order that the elongating polymerase I molecule will act as a "snowplow" and force off transcription factor(s) (Upstream Binding Factor (UBF) and possibly other factors) that have bound to the enhancer region, thus freeing up these factors for use at the gene promoter (Reeder, 1989). The elongating polymerase I molecule, having completed its function of stripping off transcription factors from the enhancer region is then terminated at the promoter proximal terminator,  $T_0$ , so as to avoid the same fate befalling the stable transcription complexes bound to the gene promoter (Bateman and Paule, 1988; Proudfoot, 1986).

While the available evidence seems to support the "snowplow" model (Grimaldi *et al.*, 1990; Pikaard *et al.*, 1990; Henderson *et al.*, 1989; Labhart and Reeder, 1989; Luccinni and Reeder, 1989; Pikaard *et al.*, 1989; Cassidy *et al.*, 1986; Busby *et al.*, 1983; Reeder *et al.*, 1983), the matter, at least in mouse, is not settled. This is because less than 1/25 of the IGS has been sequenced and characterized. What additional structures will be found and what their functions will be when the IGS is fully characterized are unknown.

Directly upstream of the gene promoter is the enhancer (ENH) region of the gene repeat. This region has been cloned and sequenced and has been found to consist of 13 tandem copies of a 135 bp subrepeating element (Kuehn and Arnheim, 1983). These enhancer regions share many of the same characteristics of polymerase II enhancers (Khoury and Grussl, 1983). They enhance

transcription from the gene promoter in a dose dependent manner (Labhart and Reeder, 1984; Reeder *et al.*, 1983; Moss, 1983), are relatively position and orientation independent (Labhart and Reeder, 1984), and can enhance the transcription of more than one promoter that is located in tandem on a single plasmid. (Busby and Reeder, 1983).

The 135 bp repeats of the enhancer region have been shown to bind the polymerase I transcription factor UBF (Pikaard *et al.*, 1990). UBF also binds in the gene promoter region, and is assumed to be required for polymerase I initiation (Schnapp and Grummt, 1991; Sollner-Webb and Mougey, 1991; Pikaard *et al.*, 1990; Reeder, 1990; Bell *et al.*, 1988).

The enhancer region is thought to function by acting as a sink for transcription factors that are required for the formation of a stable initiation complex (Reeder, 1989). When two plasmids, one with many enhancer elements, and one with few, are allowed to compete *in vitro* or *in vivo* against each other, the plasmid with more enhancer elements is preferentially transcribed (Labhart and Reeder, 1984). This dominance phenomenon can be overcome by adding additional transcription factors (eg. UBF), suggesting that the enhancer elements are competing for some limiting factor (probably UBF; Pikaard *et al.*, 1989).

Enhancers also show the ability to compete *in trans*. One plasmid with a promoter and a few enhancer repeats will show diminished transcription when a second plasmid containing only enhancer elements and no promoter is added to the reaction system (Labhart and Reeder, 1984; Reeder *et al.*, 1983). This *trans* competition is also consistent with the idea that a scarce factor is being sequestered from availability to a promoter by binding to an enhancer element.

The external transcribed spacer (ETS) region of the rRNA gene contains the promoter proximal terminator,  $T_0$ , the gene promoter, and about 3.0 kb of rRNA transcript that is clipped off of the 5' end of the primary 45S transcript as the molecule is matured to the 18S, 5.8S, and 28S rRNA molecules (Kass and Sollner-Webb, 1990; Mishimoto *et al.*, 1988; Nashimoto *et al.*, 1987; Craig *et al.*, 1987; Kass *et al.*, 1987; Grummt *et al.*, 1986; Henderson and Sollner-Webb, 1986; Mishima *et al.*, 1985).

The  $T_0$  terminator, sometimes called the failsafe terminator, is located at the 5' end of the ETS region at position -169 relative to the transcription start site.

The T<sub>O</sub> terminator has also been shown to cause elongating polymerase I molecules to release their nascent transcripts and to release from the template (Grummt *et al.*, 1986; Henderson and Sollner-Webb, 1986). The position and function of the T<sub>O</sub> terminator appears to be closely tied to the actions of the spacer promoter, the enhancer, and the downstream gene promoter. It is possible that the positioning of the T<sub>O</sub> terminator 169 bp upstream of the promoter transcription start site is very important. If the T<sub>O</sub> were closer to the gene promoter, it might allow upstream originating polymerase I molecules to interfere with the stable initiation complex formed on the gene promoter. (Henderson *et al.*, 1989). If it was located further upstream, the efficiency of the proposed snowplow effect of Reeder might be reduced (Reeder, 1989).

The gene promoter is the site of *de novo* transcription initiation. It is about 150 bp long, and generally divided into a downstream 'core' domain (~-35 to ~+9 relative to the start site of transcription; Tower *et al.*, 1986), and an upstream stimulatory domain (~-200 to ~-100; Clos *et al.*, 1986; Skinner *et al.*, 1984; Wilkinson *et al.*, 1983).

The terminology of factors that bind to the promoter is in flux. Each laboratory has developed a terminology of its own in dealing with these factors. Because different purification schemes and different experimental systems are used, it is not always a simple task to determine if one lab's factor is equivalent to another lab's factor. What seems to have emerged is that a factor termed UBF (as coined by the Tjian lab) (Bell *et al.*, 1988) binds to both the enhancer repeats and the gene promoter (Sollner-Webb and Mougey, 1991). In order for a stable pre-initiation complex to form, an additional factor, termed 'Factor D' (Sollner-Webb and Mougey, 1991), which confers species specificity, is required to bind to the promoter region (Schnapp *et al.*, 1990; Miesfeld *et al.*, 1984). A third factor, 'Factor C' (Sollner-Webb and Mougey, 1991), along with the polymerase is required for the polymerase-transcription complex to initiate (Sollner-Webb and Mougey, 1991; Reeder, 1990; Tower and Sollner-Webb, 1987; Tower and Sollner-Webb, 1986).

The formation of the UBF-Factor D pre-initiation complex is probably the slow step in the transcription reaction (Schnapp and Grummt, 1991). Once the complex is formed it has been estimated that at least 20 rounds of initiation can occur before the complex disassociates (Gokel *et al.*, 1990; Henderson *et al.*,

1989; Wandelt and Grummt, 1983). This suggests, from a kinetic point of view, that the cell would be well-served to protect the pre-initiation complex from disruption by a promoter occlusion event. Conceivably, for every promoter occlusion event the cell could prevent by terminating polymerase at  $T_1$ - $T_8$  or at  $T_0$ , the cell could gain up to 20 rounds of initiation. If maximal transcription of ribosomal DNA is the goal, then this might be an effective strategy for the cell to pursue.

Once the polymerase has transcribed the entire ETS region, the actual structural 18S, 5.8S, and 28S genes are then transcribed. The entire ETS and coding region is termed the primary 45S transcript. The 45S transcript is about 14.5 kb long, of which about 3.2 kb is ETS. The remaining 11.3 kb is composed mostly of the structural genes interspersed with two portions of transcribed spacer DNA surrounding the 5.8S gene and a short external transcribed spacer immediately downstream of the 3' end of the 28S gene (Sollner-Webb and Mougey, 1991).

Processing of the 45S transcript takes place rapidly, with the initial clipping of the primary transcript at about position +650. The event includes a endonucleolytic cleavage followed by a rapid degradation of the upstream fragment. (Craig *et al.*, 1987; Kass *et al.*, 1987). After additional processing, the resulting equimolar 18S, 5.8S, and 28S rRNAs are then incorporated into ribosomal subunits in the nucleus, and transported out into the cytoplasm where they are assembled into active ribosomes (Wool, 1979).

One of the predictions that Moss's 'read-through enhancement' model and Reeder's 'snowplow' model makes is that there should be low levels of transcription in the ENH region and no transcription in the region between  $T_0$  and the gene promoter. We decided to test these predictions by using nuclear run-on assays. We detected low but measurable transcription in the enhancer region. The origin of this transcription is unclear, as we detected transcription that had initiated at some point upstream of the spacer promoter. We could not detect any transcription between  $T_0$  and the +1 transcription start site.

We also observed a disparity in transcription between the ETS region and the 28S coding region. When we normalized our probes for nucleotide content, our 10 minute nuclear run-on assays indicated that the majority of RNA made from the ETS region is degraded rapidly.

### Regulation of rRNA transcription.

Ribosome biosynthesis is tightly coupled to the growth rate of the cell (Tushinski and Warner, 1982). Cells that are in a growth state rapidly synthesize ribosomes to meet the increased protein synthesis demands. During times of growth, the pool of free ribosomes, those not translating mRNAs, is low, indicating that virtually all ribosome are actively translating protein. (Nomura *et al.*, 1984). The transcription of rRNA is coordinated with ribosome biosynthesis, and under growth conditions is a factor in ribosome biosynthesis regulation (Nomura *et al.*, 1984). This is because the rate of accumulation of rRNA into ribosomes closely parallels the transcription rate, indicating that rRNA transcription may be a limiting factor (Freid and Warner, 1984; Meyuhas, 1984; Nomura *et al.*, 1984; Warner *et al.*, 1980). This, then, suggests that under normal growth conditions that the regulation of rRNA transcription plays a role in the ability of a cell to assemble ribosomes and, ultimately, the rate at which the cell can grow. This line of reasoning is further strengthened by the observation that at low growth rates the pool of free ribosomes grows, indicating that transcription of rRNA is not a limiting factor (Nomura *et al.*, 1984).

The pathways through which rRNA transcriptional regulation occur are not known. There are, however, pathways that have been partially elucidated for the transcriptional regulation of mRNAs and tRNAs. One pathway that has been reasonably well characterized is the receptor-triggered hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP<sub>2</sub>) (Berridge, 1987; Bell, 1986; Majerus *et al.*, 1986; Berridge, 1985; Hokin, 1985). The general schematic of the pathway is shown in Figure 2 A. An extracellular agent binds a cell surface receptor which activates phospholipase C. Phospholipase C hydrolyzes PIP<sub>2</sub> to yield inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> causes the release of Ca<sup>2+</sup> from the endoplasmic reticulum. Calcium has long been recognized as having a role as a second messenger in regulatory events of the cell, either via other proteins such as calmodulin or by directly binding to target proteins. DAG acts by activating Protein Kinase C (PKC). PKC is known to phosphorylate serines and threonines on many target proteins, thereby activating or inactivating these proteins (Ralph *et al.*, 1990; Martelly and Castagna, 1989).

Figure 2. Regulatory pathways involving protein kinase C, diacylglycerol, and insulin.

(A). Structure of A23187 and pathways involving phosphatidyl inositol 4,5-bisphosphate (PIP<sub>2</sub>) hydrolysis to yield inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> causes the release of calcium from the cell's internal stores, which results in an increased cytosolic concentration of calcium. The increased calcium level is thought to activate other proteins or to bind directly to target proteins, thereby producing a cellular response to the external signal. DAG acts by activating protein kinase C. Protein kinase C is thought to then phosphorylate target proteins, thereby facilitating a cellular response to an extracellular signal.

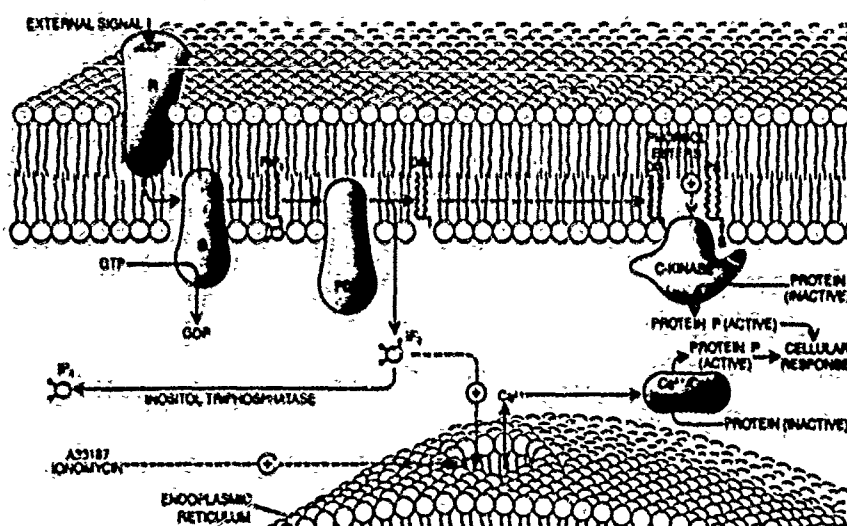
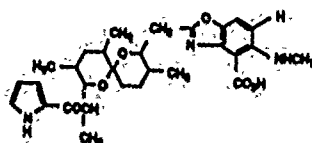
(B). The structure and sequence of bovine insulin. Insulin binds specifically at physiological concentrations to the insulin receptor. The receptor autophosphorylates and thereby activates its tyrosine kinase domain. The tyrosine kinase domain is then thought to phosphorylate other target proteins, which ultimately produce a cellular response to an external signal.



Figure 2

A.

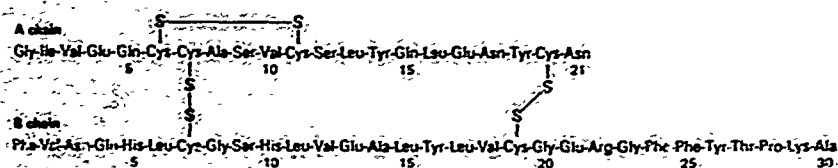
Antibiotic A23187



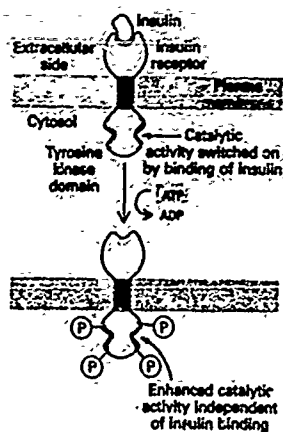
Berridge, M. J. Scientific American. 253,4, 147 (1985).

Figure 2 (continued)

B.



Amino acid sequence of bovine insulin.



Activation of the tyrosine kinase activity of the insulin receptor by autophosphorylation.

Stryer, L. In Biochemistry, 3rd Edition. ( W.H. Freeman & Co. NY. 1988).  
 pp 23 + 997.

An interesting aspect of this pathway is that it can be manipulated by agents that resemble or interfere with the various components of the pathway. One agent that mimics the action of PKC is the tumor promoting phorbol ester, 12-tetradecanoylphorbol-13-acetate (TPA). TPA resembles DAG in structure, and substitutes for DAG as a long lived activator of PKC (Fornier and Murray, 1987; Thomas, 1987; Berridge, 1985). A second agent which raises cytosolic calcium levels is the calcium ionophore A23187. A23187 permeabilizes a cell's internal and external membranes to calcium, with a net result that the cell's internal stores of calcium are released (Pressman, 1976). This release of  $\text{Ca}^{2+}$  ions is thought to then mimic the action of  $\text{IP}_3$ .

Another agent that can effect the growth rate of a cell, and by correlation, the rate of rRNA transcription is Fetal Bovine Serum (FBS). FBS is a common media supplement that is used to ensure establishment and/or growth of tissue culture cell lines (Kingston, 1989). The amount of FBS that is added to a cell line's media commonly varies between 10-20% (vol/vol) (Hay *et al.*, 1988). The FBS is an undefined mixture of peptides that is extracted from fetal cows (Kingston, 1989). The mode of action of FBS is not known, however, it is assumed to supply all required growth hormones, peptides, and factors in a 'shotgun' approach.

There is a rough correlation between the amount of FBS added to a cell line and the growth rate of that particular culture. In general, the doubling time of a tissue culture will lengthen with the addition of less FBS and decrease with the addition of more FBS, within limits. For instance, we have found that mouse macrophage cells which are normally supplemented with 10% FBS have a doubling time of about 48 hours. If the FBS supplementation is reduced to 2%, the cells essentially enter a stationary growth phase, and doubling time is on the order of two weeks.

Insulin is a peptide hormone that has wide ranging effects on target cells. One effect of insulin that is germane to this study is the promotion of anabolic processes in the target cell (Granner and O'Brien, 1991). When insulin binds to the cell surface receptor, the receptor autophosphorylates (Rosen, 1987; Gammeltoft and Van Obberghen, 1986). The phosphorylated receptor is able to phosphorylate tyrosine residues on its target peptides. One of the effects of this tyrosine kinase activity is the stimulation of glycolysis. Glycolysis ultimately

leads to the synthesis of macromolecules, which are essential for the growth of the cell (for an overview of glycolysis see Stryer, 1988).

Because insulin stimulates anabolic processes, and because it is known to influence mRNA transcription (Granner and O'Brien, 1991), it would seem likely that ribosome biosynthesis might be in some fashion regulated by insulin. If this is the case, then rRNA transcription would also probably be regulated by insulin either directly or as an indirect result.

Serum, insulin, A23187 and TPA stimulate tRNA gene expression (Garber *et al.*, 1992; Weber *et al.*, 1991). These same agents also stimulate mRNA gene expression (for example: Granner and O'Brien, 1991; Dean *et al.*, 1990; Martelly and Castagna, 1989; Kozo *et al.*, 1986). However, while there is documentation that rRNA transcription can be stimulated by these agents, the dogma still persists that transcription of rRNA genes does not respond rapidly to extracellular signals.

We were interested in examining what pathways might be involved in the regulation of rRNA transcription. In order to determine what pathways might be involved, we decided to treat J774.A1 mouse macrophage cells with TPA, A23187, insulin, or serum. We assessed the ability of these agents to stimulate rRNA transcription in cells that were growing in a 'normal' growth environment and also in a restricted growth environment.

In order to assess the possibility of rapid stimulation of rRNA transcription, we treated both cells growing in 10% FBS and cells shifted to 2% FBS for 48 hours with additional serum, reasoning that probably some factor in the serum supplement would stimulate the transcription of rRNA. We also treated both 10% and 2% cells with insulin to see if a rapid stimulatory response could be elicited for rRNA transcription. We reasoned that insulin was a good choice as a peptide hormone because of its involvement in the anabolic processes of the cell. Accordingly, we treated 10% and 2% FBS cells with insulin and assayed for a rapid rRNA transcriptional response. Further, we treated 10% and 2% FBS cells with TPA and A23187 to see if either DAG or PKC could be implicated in rRNA transcription regulation.

We observed that serum, insulin, TPA, and A23187 all stimulated rRNA transcription as judged by nuclear run-on assays. Further, the transcriptional stimulation was seen as increased transcription initiating at the gene promoter

and possibly at the spacer promoter. Interestingly, we saw no evidence of increased transcription in the enhancer region. The increases we saw took place both in cells that were growing in 10% FBS and in cells which had been shifted to 2%FBS for 48 hours, with 10% FBS cells showing a greater ability to increase rRNA transcription than 2% cells. The responses in rRNA transcription occurred rapidly, with TPA producing a measurable response after 15 minutes of treatment. Insulin and A23187 produced measurable responses after 30 minutes of treatment.

## MATERIALS AND METHODS

### Cell cultures and maintenance of cell cultures.

J774.A1 mouse monocyte macrophage cells (ATCC TIB 67) were grown in DMEM high glucose media supplemented with 10% (vol/vol) heat inactivated fetal bovine serum (FBS) (Gibco), 200 mM Glutamine (Sigma), and 50 U/ml penicillin,  $5 \times 10^{-5}$  g/ml streptomycin and  $1 \times 10^{-4}$  g/ml neomycin (Sigma). Cells were grown in T-25, T-75, or T-150 (Corning) disposable flasks in a 95%/5% air/CO<sub>2</sub> environment at 37°C.

When cells reached approximately 80% confluence, they were scraped with a rubber policeman, resuspended in fresh DMEM/FBS media, and split into new flasks at a 1 : 3 or 1 : 4 ratio. During normal growth, media from the flasks was removed and replaced with fresh media every 3-4 days.

### Cell labeling and RNA isolation techniques.

J774.A1 cells were plated in 60 mm plastic petri dishes (Falcon) in DMEM media containing either 10% or 2% FBS (hereafter called 10% FBS cells or 2% FBS cells). Cells were incubated overnight for 24 hours at 37°C in a 95%/5% air/CO<sub>2</sub> environment to allow adherence to the dish. After adherence, cells were washed 2X with phosphate buffered saline (Sigma) and additional DMEM containing 10% or 2% FBS was added. Cells were treated with insulin (courteously provided by Eli-Lilly) at a concentration of either  $6.7 \times 10^{-8}$  M,  $6.7 \times 10^{-9}$  M, or  $6.7 \times 10^{-10}$  M and incubated for either one hour or three hours at 37°C. [<sup>32</sup>P]-orthophosphate (Amersham) was added (10-50 µCi/dish) at the same time as insulin for the one hour incubation samples or for the last hour of the three hour incubation samples.

In serum addition experiments, cells that were shifted to 2% FBS for 48 hours were given additional FBS to yield a 10% FBS concentration (vol/vol) overall. Cells continually growing in 10% FBS were treated with additional FBS to increase FBS to 15% (vol/vol) overall. Cells given additional serum were

allowed to incubate for one or three hours at 37°C. [ $^{32}\text{P}$ ]-orthophosphate was added in the same manner as for insulin treated cells.

After the incubation was complete, radioactive media was aspirated off, cells were washed 2X with PBS, and one ml of 2% FBS DMEM or 10% FBS DMEM was added to the 2% FBS or 10%FBS cells, respectively. Cells were scraped off the dish with a rubber policeman and the cells and media were placed in 15 ml conical tubes (Sardstedt). Each dish was washed and scraped again with an additional one ml of media, which was pooled together with the first scraping of cells and media. Cells were pelleted in a table top centrifuge (500xg, 2 minutes). The supernatant was decanted and total RNA was extracted from the cells by the method of Cathala *et al.* (1983).

Cell pellets were disrupted by the addition of 500  $\mu\text{l}$  of lysis buffer [5 M guanidine thiocyanate (Fluka), 10 mM EDTA, pH 8.0 (Fisher) 50 mM tris-HCl, pH 7.5 (Sigma)] and vortexing. 3.5 ml of 4 M LiCl (EM Science) was added and samples were stored overnight at 4°C. The following day samples were centrifuged (4°C, 12,000xg, 90 minutes). Supernatant was discarded and 3.5 ml of 3 M LiCl was added to each sample. Samples were vortexed to resuspend pellets and centrifuged (4°C, 12,000xg, 60 minutes). After centrifugation, the supernatant was decanted and discarded and pellets were resuspended in one ml of RNA solubilization buffer (0.1% SDS, 1mM EDTA, 10 mM tris-HCl, pH 7.5) Samples were frozen at -20°C and then vortexed as they melted. Samples were extracted 2X with 500  $\mu\text{l}$  phenol (Clonetech) (equilibrated to approximately pH 7.0 with 50 mM Tris, 1mM EDTA) and 500  $\mu\text{l}$  of  $\text{CHCl}_3$  : Isoamyl alcohol (24 : 1), and 2X with 1ml of  $\text{CHCl}_3$  : Iso amyl alcohol (24 : 1). RNA was precipitated at -20°C overnight in the presence of 0.05 volumes of 75 mM  $\text{NH}_4\text{OAC}$  and 2.5 volumes of 100% ethanol. Samples were then centrifuged (4°C, 7,000xg, 30 minutes), washed with one ml of 95% ethanol, and dried.

Labeled RNA was resuspended in DEP-treated water, denatured by glyoxylation (Kodak)(McMaster and Carmichael, 1977), and resolved on 1.0% agarose- $\text{NaH}_2\text{PO}_4$  gels (BRL)(Maniatis *et al.*, 1982) at 7 v/cm. Gels were stained with ethidium bromide (1 $\mu\text{g/ml}$ ) for 15 minutes, photographed under uv light (Polaroid Type 55 Film), dried, and placed in contact with X-ray film (Kodak XAR-5).

The negative image produced when photographed under uv light allowed for normalization of loading by comparing unlabeled 18S and 28S rRNA bands of treated samples against the untreated control.

#### Plasmid constructions.

Plasmids pMrA and pMrD (Kuhn and Grummt, 1987) were kind gifts of Dr. Ingrid Grummt. Plasmid pl19 and pl23 (Arnhim, 1979) were kind gifts of Dr. Norman Arnheim. Plasmids pUC9, pUC19, and pUC18, T4 DNA ligase, all restriction enzymes and molecular weight markers were purchased from Gibco-BRL. Lambda phage DNA was purchased from NEN. Klenow fragment was purchased from Amersham. Sequencing reagents and enzymes and primers were purchased from USB. *E. coli* strain DH5-alpha was a kind gift from Dr. David Galas. Growth media was purchased from DIFCO. Ampicillin was purchased from Sigma. pGEM3Z and Riboprobe Gemini System were from Promega. dNTPs were from Pharmacia. GeneClean and Mermaid kits were from Bio 101. Large scale DNA preparations were purified using PZ523 columns from 3prime->5prime.

In general, all digestions, fill in reactions, ligations, fragment purifications, sequence determinations and large scale DNA preparations were performed according to the manufacturers specifications. DH5-alpha was transformed using the Hanahan method (Hanahan, 1986) and grown on LB agar plates containing 60 mg/ml ampicillin. Colonies were screened using Dr. Richard Deonier's "toothpick" assay (personal communication). Briefly, a transformed colony is streaked to about a 1 cm x 1 cm size on a LB/AMP plate and allowed to grow overnight. A sterile toothpick is used to remove a small quantity of the colony and resuspended in 75 µl of TAE buffer, pH 8.2. Twenty µl of lysis solution [2% SDS, (vol/vol) 25% glycerol (wt/vol), 0.1 M EDTA pH 8.5, and 0.025% bromophenyl blue (wt/vol)] was added to the sample. The sample was heated to 70°C for 10 minutes, and centrifuged at 10,000 rpm for 15-30 minutes (room temp). A 20 µl sample of the resulting supernatant was then loaded on a 1.0% agarose mini-submarine gel (TAE buffer) and the gel was run at 8 volts/cm for approximately 40 minutes. Plasmid bands were visualized by staining with 1 µg/ml ethidium bromide and viewing under uv light. Plots of mobility versus log of molecular weight were constructed to determine size of plasmids. Colonies



which contained plasmids which conformed to the expected size were grown overnight in 1-2 liter of LB/AMP broth with shaking at 37°C. Crude DNA lysates were obtained from the bacteria using the alkaline lysis method (Ausubel *et al.*, 1989). pZ523 spin columns (3prime-->5prime) were used for final DNA purification. DNA concentration and purity was then determined using A<sup>260</sup>/A<sup>280</sup> spectrophotometry. The first 50-100 nucleotides of both ends of the plasmid were then determined using a Sequenase Version 1.0 (USB) sequencing kit with forward and reverse primers. Plasmid identity was then verified by comparing to published sequences in Genbank.

Plasmid pI19 contains a 4.8 kb fragment that includes the mouse 28S ribosomal gene in a pBR322 vector. A 1.3 kb Bam HI-Bam HI fragment of the 28S gene was digested, purified, and ligated into the Bam H1 site of pUC9 to produce pMGC28.

Plasmid pI23 contains a 3.2 kb Sal I-Sal I fragment which includes most of the mouse ribosomal external transcribed segment in a pBR322 vector. pI23 was digested with Sal I to liberate the 3.2 kb fragment. The fragment was then ligated into the Sal I site of pUC9 to produce pMG23.

Plasmid pMGC contains a 998 bp lambda Eco RI-Sal I fragment ligated into the polylinker region of pGEM3Z.

Plasmid pMrD is a pUC9 vector that contains the 1.7 kb Sal I-Sal I enhancer region fragment of the mouse ribosomal gene.

Plasmid pMrA is a pUC9 vector that contains the 3.8 kb Eco RI-Sal I fragment of the mouse ribosomal Intergenic Spacer Region which is located immediately upstream of the enhancer region.

Plasmid pMGTP contains the 169 bp Sal I - Rsa I fragment that includes sequences from -168 to +1 of the mouse ribosomal promoter region. The Sal I - Rsa I fragment is ligated into a pUC 18 Sal I - Rsa I digested vector.

#### Nuclei isolation and run-on transcription.

Nuclei were isolated for *in vitro* transcription by a modification of the methods of Hewish and Burgoyne (1973) and Schibler *et al.* (1983). J774.A1 macrophage cells were either grown continuously in DMEM/10% FBS or shifted from growth in DMEM/10% FBS to DMEM/2% FBS for 48 hours. All cells were

growing in logarithmic phase prior to starting the experiments. Eight to ten T-150 flasks of cells, which were approximately 80% confluent, were scraped with a rubber policeman and pooled together. Pooled cells were split into equal sized aliquots: control, TPA, A23187, insulin, or serum. Cells were treated as follows: control - no additions. TPA (Sigma)- addition of  $5 \times 10^{-8}$  M TPA for 15 minutes. A23187 (Calbiochem) - addition of  $0.5 \times 10^{-6}$  M A23187 for 30 minutes, insulin - addition of  $6.7 \times 10^{-9}$  M insulin for 30 minutes. Serum - 10% FBS cells were boosted to 15% FBS (vol/vol). 2% FBS cells were boosted to 10% FBS (vol/vol). Serum incubations were for 3 hours. All incubations were at room temperature with mild shaking.

After treatment, cells were pelleted ( $4^{\circ}\text{C}$ , 500xg, 10 minutes), and the supernatant was discarded. From this point, all manipulations occurred on ice or at  $4^{\circ}\text{C}$ . Cells were resuspended in 1/10 original volume of 0.3 M sucrose in buffer A [60 mM KCl, 125 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM beta-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 15 mM hepes (pH 7.5)]. NP-40 was added to the cells to a final 0.2% concentration (vol/vol). Lysis of cells occurred during a five minute incubation on ice. In 50 ml polypropylene tubes, the lysate was layered over a 10 ml pad of 20% Sucrose in buffer A. Nuclei were pelleted in a swinging bucket rotor ( $4^{\circ}\text{C}$ , 1,300xg, 10 minutes). The resulting supernatant was decanted and discarded. The nuclei pellet was resuspended in 5 ml of 0.3 M sucrose in buffer A. To this resuspension, 25  $\mu\text{g/ml}$  of RNase A was added and the samples were incubated on ice for 30 minutes. After incubation, the samples were layered over a 10 ml pad of 20% sucrose in Buffer A and centrifuged to recover nuclei ( $4^{\circ}\text{C}$ , 1,300xg, 10 minutes). The resulting supernatant was decanted and discarded. The pelleted nuclei were gently resuspended in 5 ml of 0.3 M sucrose in Buffer B [60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM beta-mercaptoethanol, 0.1 mM EGTA, 0.1 mM EDTA, 15 mM hepes (pH 7.5)] and layered over a 5 ml pad of 20% sucrose in Buffer B and centrifuged ( $4^{\circ}\text{C}$ , 1,300xg, 10 minutes). The supernatant was decanted and discarded. The pelleted nuclei were gently resuspended in one ml of 0.3 M sucrose in Buffer B, and an aliquot was counted on a hemacytometer after staining with 0.4% crystal violet in 0.7 M citric acid. After counting, nuclei were pelleted ( $4^{\circ}\text{C}$ , 1,300xg, 10 minutes) and resuspended

in nuclei storage buffer (20 mM Tris-HCl, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 50% glycerol (vol/vol) and 0.125 mM PMSF) and  $1 \times 10^7$  nuclei per sample were aliquoted into pre-chilled 15 ml polypropylene tubes. Nuclei were then stored at  $-70^{\circ}\text{C}$ , where they remained transcriptionally active for longer than six months.

Nuclear run-on reactions were carried out using a modification of the method of Gariglio *et al.* (1981). Frozen nuclei were thawed on ice and an equal volume of reaction cocktail was added to make an overall reaction mix containing: 100 mM Tris-HCl, pH 7.9, 50 mM NaCl, 0.4 mM EDTA, 0.1 mM PMSF, 1.2 mM DTT, 350 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1mg/ml heperin sulfate, 4 mM  $\text{MnCl}_2$ , 100 $\mu\text{Ci}$  [ $^{32}\text{P}$ ]-UTP (NEN 800 Ci/mmol), 1 mM GTP, 1 mM ATP, 1mM CTP, 25% glycerol (vol/vol), 10 mM creatine phosphate, 320 U RNasin RNase Inhibitor (Promega), 100  $\mu\text{g/ml}$  alpha-amanitin (Sigma).

The reaction cocktail was allowed to equilibrate one minute on ice before transfer of the sample to a  $26^{\circ}\text{C}$  waterbath for 10 minutes. At the end of the 10 minute run-on reaction, the nuclei are disrupted and RNA was extracted according to a modification of the Chomczynski and Sacchi method (1987). After denaturation of protein by solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M beta- mercaptoethanol) and vortexing, water saturated phenol and chloroform : iso-amyl alcohol (24 :1) and 2 M sodium acetate, pH 4.3 were added. After a 15 minute incubation on ice and phase separation by centrifugation (room temp, 500xg, 5 minutes) the aqueous phase was removed and saved, and the organic phase was back extracted with one ml of solution D. The back-extraction was phase separated as above, and the aqueous phase was removed and pooled with the first aqueous phase. RNA was precipitated by the addition of 1.5 volumes of isopropanol and incubating at  $-20^{\circ}\text{C}$  overnight. The RNA was sedimented ( $4^{\circ}\text{C}$ , 10,000xg, 20 minutes) and the resulting pellet was resuspended in 0.3 ml of solution D, transferred to a 1.5 ml microfuge tube, and precipitated with one volume of isopropanol at  $-20^{\circ}\text{C}$  overnight. The RNA was pelleted ( $4^{\circ}\text{C}$ , 10,000xg, 20 minutes), washed with 95% ethanol, and briefly air dried before resuspension in one ml of DEP-treated water. A 5  $\mu\text{l}$  aliquot of the resuspended RNA was counted in 5 ml of EcoScint scintillation fluid in a Beckman LS 7000 Scintillation Counter.

#### Determination of incorporation of [ $^{32}$ P]-UTP.

After the addition of solution D and the phenol : chloroform mix, a 5  $\mu$ l aliquot was spotted on quadruplicate Millipore HAWP 0.45  $\mu$ m filters. Two of the filters were washed one time each with 5 ml of cold 10% TCA, and two times each with 5 ml of cold 5% TCA and allowed to air dry. The other two filters were allowed to air dry without washing. All filters were then placed into individual scintillation vials and 5 ml of EcoScint scintillation fluid (National Diagnostics) was added to each vial. Samples were counted on program #10 ( $^{32}$ P) of a Beckman LS 7000 Liquid Scintillation Counter.

#### Hybridizations and Autoradiography.

After labeled RNA samples were purified, they were hybridized against complementary DNA that was bound to Zeta-probe (Bio-Rad) nylon membranes. 2.5 pico-moles of DNA from clones pMrA, pMrD, pMG23, pMGC28, and pMGC, or, pMrA, pMrD, pMG23, and pMGC28, or, pMrA, pMrD, pMGTP, and pMGC28 were dot blotted to the nylon membrane using a Bio-Rad 96 well dot blotter according to the manufacturer's protocol. A single filter contained multiple dots of each plasmid to be sure that bound DNA was in excess to the nascent run-on RNA that would be used to probe the dot blots. DNA was uv crosslinked to the nylon membrane by exposing the membranes to shortwave uv light for 20 seconds (approximately 125 mW) while still wet. Membranes were then air dried and baked in a vacuum oven at 80°C for >30 minutes. The baked membranes were stored between two pieces of Whatman paper at room temperature.

Membranes were prehybridized for 2-4 hours at 42°C with gentle shaking in prehybridization solution (50% formamide (vol/vol), 5% 100X Denhardt's (vol/vol), 0.05 M NaPO<sub>4</sub>, 0.02 M tris, pH 7.5, 0.025% SDS (vol/vol), 10 mg/ml sheared salmon sperm DNA) that was made fresh before use.

Hybridization reactions occurred at 42°C with gentle rotation in 15 ml Sardstedt tubes. Five ml of hybridization solution (50% formamide (vol/vol), 15% hybridization cocktail (vol/vol) [for 30 ml: 25 ml 20X SSC, 1 ml 100X

Denhardt's, 2 ml 1M NaPO<sub>4</sub>, 10 g dextran sulfate](Wahl *et al.*, 1979), 10 mg/ml sheared salmon sperm DNA, 0.1% SDS (vol/vol) was added to each tube. Membranes to be hybridized were cut into strips 1.5 cm wide and 10 cm long, and placed into the tubes so as to adhere to one side of the tube wall. Between 5 and 40 µl of labeled RNA sample was added to each tube, depending on the specific activity of the sample as determined by counting 5 µl of the labeled RNA sample on a scintillation counter.

Hybridizations were carried out for 48-96 hours at 42°C while slowly rotating the tubes so that the hybridization solution would continually bathe the nylon membranes. After hybridization was complete, individual tubes were treated with 150 µg/ml of RNase A for >30 minutes at 42°C with gentle rotation.

Upon completion of RNase A treatment, membranes were removed from tubes, washed three times at room temperature for 5 minutes in 2X SSC/0.1% SDS, and once at 55°C for 30 minutes in 0.2X SSC/0.1% SDS. Membranes were then blotted with Whatman paper, and allowed to completely air dry. Typical cpm were between 150-300 cpm per membranes as judged by a hand held geiger counter. Dried membranes were arranged for autoradiography and exposed to Kodak XAR-5 X-ray film in cassettes with Dupont Cronex intensifying screens for 72-96 hours at -70°C. In cases where cassettes with intensifying screens were unavailable, X-ray film was exposed to membranes at room temperature for 10-14 days.

#### Densitometry of autoradiographs.

Quantitation of dot blot autoradiographs was accomplished by using an LKB Laser Densitometer. Autoradiographs were calibrated by first scanning across an optically opaque tape. The tape produced a peak that was both consistent in height and width and in integrated area from autoradiograph to autoradiograph. The autoradiographs were then scanned and compared by determining the relative area under each peak. Both the internal LKB integration program and cutting and weighing was used to quantitate each peak.

### Statistical analysis.

Statistical analysis was performed using the one-tailed  $t$  Test. (Hamburg, 1983) Multiple hybridizations of a single nuclear run-on were averaged together. Standard Error (S.E.) estimates were determined for bar graphs showing data from nuclear run-on reactions conducted in the presence of alpha-amanitin.

### Cell growth rate determination.

Five  $\times 10^5$  J774.A1 cells that were growing in 10% FBS were plated in T-25 flasks (Corning) in either 10% FBS or 2% FBS. Multiple flasks were plated on day one in this manner. On each succeeding day, one flask of 10% FBS cells and one flask of 2% FBS cells were scraped and counted using a hemacytometer (Patterson, 1979) and Trypan blue stain. Only cells which excluded the Trypan blue dye were included in the cell totals. Total cells per flask were counted per flask and graphed against time in order to determine cell doubling times.

## RESULTS

### rRNA synthesis in rapidly growing and slowly growing mouse J774.A1 Macrophage cells is responsive to external agents.

The idea that ribosomal RNA (rRNA) transcription is unresponsive to cellular growth conditions has been challenged only in the last few years. It was long assumed that rRNA, which makes up the majority of all cellular RNAs, exists in the cell as a large pool and that the rate of synthesis of rRNA is relatively immune to changes. Indeed, numerous investigators continue to use the rate of synthesis of rRNA as an internal control, against which they measure changes in the pool size of other genes.

Work by investigators in the last decade suggests that while the rRNA pool size is relatively stable, the transcription of rRNA is much more responsive to external signals than was once thought. We were interested in understanding what mechanisms might be responsible for upregulating or downregulating the transcription of rRNA, and, if possible, to quantitate the stimulation that occurs in mammalian cells.

It has been demonstrated by several investigators in several different systems that genes transcribed by RNA polymerase II (pol II) can be upregulated and downregulated by factors such as serum, insulin, tumor promoting agents, and agents that act on calcium regulation. Work in this lab on *Drosophila* Schneider Line-2 cells (Vallett *et al.*, submitted; Weber *et al.*, 1991; Yamamoto *et al.*, 1988) and non-mitotic cells from the *Drosophila* male accessory glands (Weber *et al.*, 1991) suggests that these types of agents also stimulate rRNA transcription under certain conditions. These observations led us to screen several mouse lines by analyzing the growth rate characteristics and the incorporation of [ $^{32}\text{P}$ ]-orthophosphate into precursor and mature ribosomal RNAs of cells that have been treated by these reagents.

Serum supplementation of media is an absolute requirement of most mammalian cell culture lines. Typically, lines are grown in DMEM media supplemented with FBS. This serum is an undefined mixture containing

polypeptides which have proven useful in the establishment and maintenance of most cell lines. The mode of action of cell line maintenance by serum is unclear for most cell lines, however, it is widely thought that the serum supplies the necessary growth factors and signaling agents required for cell line viability in a "shotgun" type approach.

The cell line that we chose was the J774.A1 mouse macrophage line (ATCC TIB 67). We chose this line for three primary reasons. First, macrophage lines are differentiated lines. This allowed us to, at least to some degree, separate out developmental effects of increased rRNA transcription. Second, macrophage lines have previously demonstrated responses to stimulation by various agents (Radzioch *et al.*, 1987; Varesio *et al.*, 1984). And third, J774.A1 macrophages are a semi-adherent cells that have a relatively spherical morphology. This characteristic allows for easy growth to high titers ( $\approx 1 \times 10^6$  cells/ml).

J774.A1 cells were typically grown in high glucose DMEM medium supplemented with 200nM L-glutamine (Sigma), 50 U/ml penicillin,  $5 \times 10^{-5}$  g/ml streptomycin, and  $1 \times 10^{-4}$  g/ml neomycin (Sigma) and either 10% or 2% fetal bovine serum (FBS) (volume/volume, hereafter called 10% or 2% FBS). In order to assess the effects of FBS on the growth rate of the cells, cells in mid-logarithmic growth phase were scraped with a rubber policeman in total media and divided into two aliquots. Each aliquot was centrifuged at 500xg for 3 minutes. The cells were then resuspended in either DMEM media supplemented with 10% or 2% FBS. Aliquots were counted in the presence of trypan blue (Patterson, 1979) on a hemacytometer. Five  $\times 10^5$  cells were then plated into T-25 flasks (Corning) and incubated in a 95% air/5% CO<sub>2</sub> environment at 37°C. At 24 hour intervals the T-25 flasks were scraped with a policeman in total media and the cells were counted in the presence of trypan blue stain on a hemacytometer. Only viable cells were included in the resulting growth curves.

As can be seen in Figure 3, the cells growing in media supplemented with 10% FBS show a doubling time of about 48 hours. Cells that were shifted to media supplemented with 2% FBS show an almost flat growth rate for the first 72 hours. J774.A1 cells that are grown in the presence of 10% FBS will continue to grow rapidly, until ceasing growth at confluence after about 12 days at a density of about  $1 \times 10^7$  cells/flask (data not shown). The J774.A1 cells grown in the presence of 2% FBS show a doubling time of about 14 days. Eventually the cells

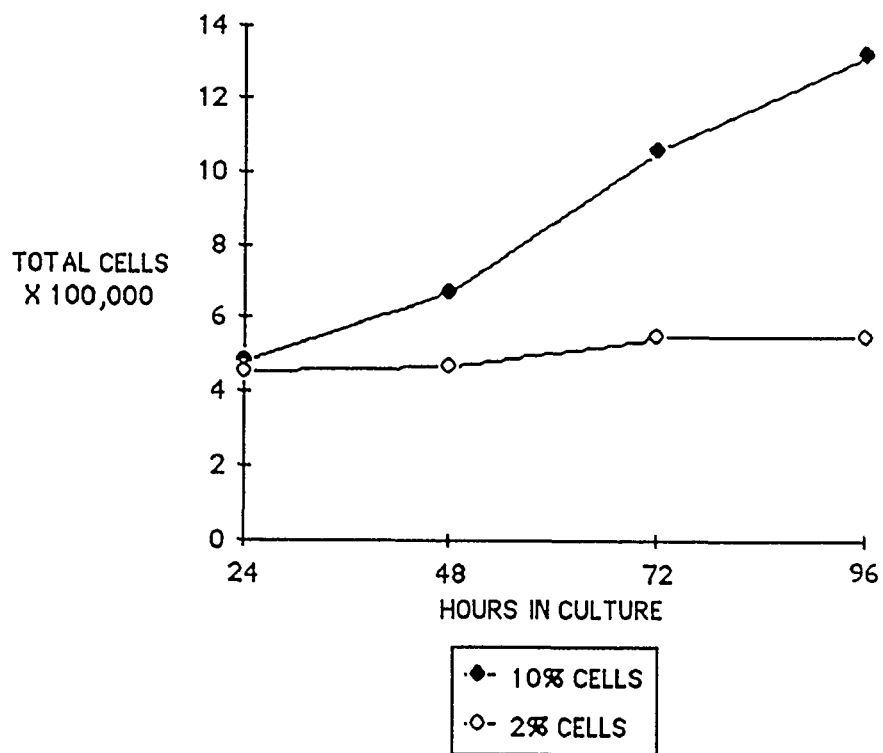


Figure 3. Cell growth curves.

Five  $\times 10^5$  J774.A1 cells in 10% FBS media were each plated into multiple T-25 flasks and allowed to continue growth in a 95%/5% air/ $\text{CO}_2$  environment. One-half of the samples had their media replaced with 2% FBS, while the other half of the samples had fresh 10% FBS added. Every 24 hours a 2% FBS and a 10% FBS sample was scraped and total cells were counted on a hemacytometer. Cells were treated with trypan blue prior to counting, and only cells which excluded the dye were included in the totals. The resulting cell counts were plotted against time. The first count took place at 24 hours, and indicated a slight decrease in cell number due to the shock of scraping and reattachment to the flask.

Figure 3

J774.A1 cell growth curves.



grown in the presence of 2% FBS will enter log-phase growth, but the deprivation of serum delays this until after day 14 (data not shown).

As a means of assessing the possible stimulatory effect of insulin on transcription of ribosomal RNA, J774.A1 cells were grown in DMEM media supplemented with 10% FBS or were shifted for 24 hours to DMEM media containing 2% FBS. These cells were then treated with varying physiological concentrations of insulin for one or three hours. [ $^{32}\text{P}$ ]- orthophosphate was either added at the same time as the insulin was added (one-hour experiments) or for the last hour of incubation time (three-hour experiments). Total RNA was then extracted, glyoxylated, and resolved on an agarose gel. RNA was visualized by staining with ethidium bromide, and visualized by autoradiography (Maniatis, 1982)(Figure 4). The cells treated with insulin show an increase in the amount of mature 28S ribosomal RNA and also an increase in the amount of unprocessed ribosomal RNA.

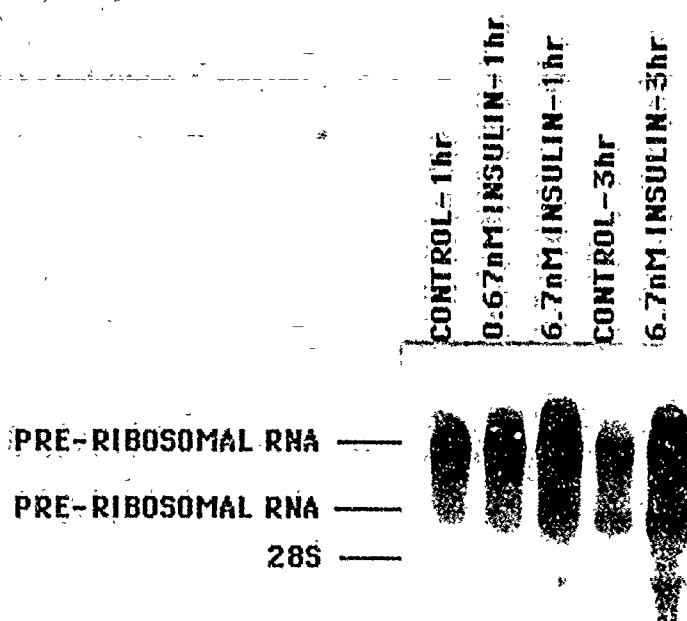
Based on these and other experiments utilizing FBS (not shown) and on other work done in the laboratory (Vallett *et al.*, submitted; Weber *et al.*, 1991), we decided to determine to what degree insulin, the calcium ionophore A23187, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), or FBS stimulate the transcription of ribosomal RNA.

The *in vivo* labeling experiments cannot distinguish the mechanism of the increase in pre-ribosomal RNA synthesis. The increase in pre-ribosomal RNA synthesis could be due to increased polymerase loading, increased transcriptional efficiency, reduced degradation or increases in the number of rRNA genes competent for transcription, to name a few. Also, *in vivo* labeling experiments give no indication of transcriptional differences occurring in different parts of the gene. In order to better address these issues, we decided to conduct nuclear run-on assays using nuclei from cells that had been either continually grown in 10% FBS or shifted to 2% FBS for 48 hours. We treated 10% FBS and 2% FBS J774.A1 cells with TPA, A23187, insulin, or serum, and then isolated the nuclei and conducted nuclear run-on reactions. We judged that nuclear run-on assays would help us determine the mechanism responsible for the increased pre-ribosomal RNA transcription observed in the *in vivo* labeling experiments.

Figure 4. Representative autoradiograph of *in vivo* labeling experiments of J774.A1 cells.

Equal numbers of J774.A1 cells grown in 10% FBS were plated and allowed to adhere to the petri dish. Cells were treated with either nothing,  $6.7 \times 10^{-10}$  M, or  $6.7 \times 10^{-9}$  M insulin for one hour or with nothing or  $6.7 \times 10^{-9}$  M insulin for three hours.  $[^{32}\text{P}]$ -orthophosphate was added at the same time as the insulin (one hour experiments), or for the third hour (three hour experiments). After incubation cells were lysed and total RNA was isolated and resolved by gel electrophoresis. Gels were stained with ethidium bromide and photographed with Kodak pos/neg film. The 18S and 28S rRNA bands on the negatives was scanned with a densitometer. These scans were used to control for loading and recovery errors (data not shown). The representative autoradiograph shows increased incorporation of label into insulin treated cells, especially in the pre-ribosomal bands.

**Figure 4.** *In vivo* labeling of J774.A1 cells.



Isolation of nuclei from J774.A1 macrophage cells by lysis with NP-40 and Sucrose gradient purification.

Nuclei were isolated for *in vitro* run-on assays by a modification of the methods of Hewish and Burgoyne (1973) and Schibler *et al.*(1983). Nuclei were isolated from log-phase J774.A1 macrophage cells that had been either growing continuously in media supplemented with 10% FBS, or that had been shifted to 2% FBS for 48 hours. These nuclei were treated with RNase A to remove endogenous RNAs. The resulting nuclei were intact, as judged by crystal violet staining (Patterson, 1979), and transcriptionally active, as judged by [ $^{32}$ P]-UTP incorporation.

Run-on transcription increases linearly over time and is sensitive to alpha-amanitin.

In order to monitor the incorporation of [ $^{32}$ P]-UTP specifically into RNA polymerase I (pol I) transcripts, and the efficiency of the run-on reaction, we conducted the reactions in the presence and absence of alpha-amanitin, a well characterized inhibitor of mammalian polymerase II and III. As can be seen in Figure 5, run-on reactions that were conducted in the presence of alpha-amanitin showed a linear incorporation rate for at least 15 minutes. After 15 minutes the incorporation rate plateaus, indicating little if any new incorporation of [ $^{32}$ P]-UTP into TCA precipitable material. Since mammalian RNA polymerases II and III are known to be sensitive to high levels of alpha-amanitin (100  $\mu$ g/ml) and that mammalian pol I is insensitive to alpha-amanitin at the same level, we concluded that the level of incorporation seen in the run-ons containing alpha-amanitin was due solely to transcription by pol I. The alpha-amanitin insensitive transcription activity varied considerably between nuclei preparations. Cells that were grown in 10% FBS showed about 44% alpha-amanitin insensitivity. Cells that had been shifted to 2% FBS showed between showed about 25% alpha-amanitin insensitivity. Other workers have reported similar levels for pol I transcription (Marzluff and Huang, 1984).

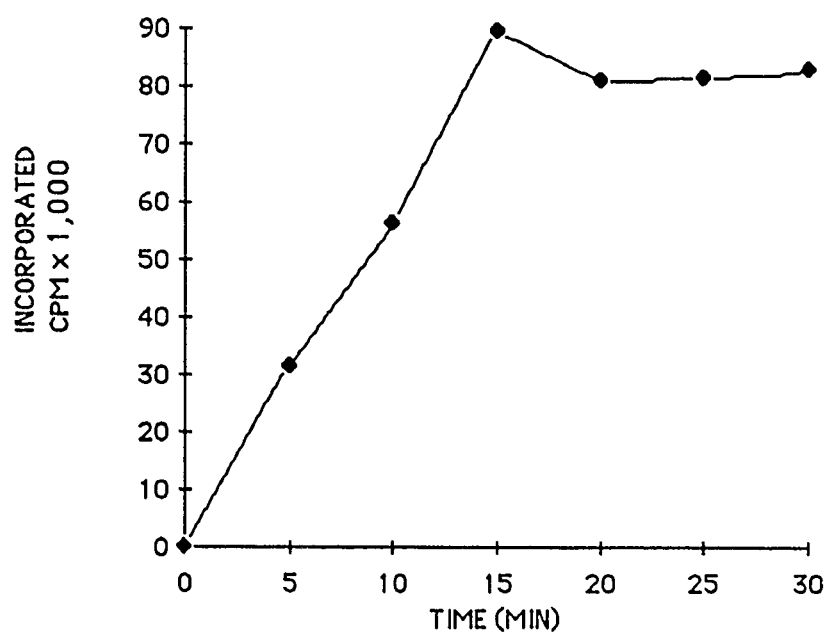
Incorporation of [ $^{32}$ P]-UTP varied between nuclei preparations. [ $^{32}$ P]-UTP incorporation was generally higher in nuclei prepared from cells grown in

Figure 5. Incorporation of [ $^{32}\text{P}$ ]-UTP into RNA by alpha-amanitin insensitive polymerase 1 transcription .

Nuclear run-on reactions using  $1 \times 10^7$  nuclei were conducted in the presence of 100  $\mu\text{g/ml}$  of alpha-amanitin. Five  $\mu\text{l}$  aliquotes of the reaction were taken periodically as the reaction proceeded. Aliquotes were counted in a liquid scintillation counter and cpm was plotted against time. The graph plots an untreated nuclei preparation from cells grown continually in 10% FBS. Label was incorporated in a linear fashion for at least the first 10 minutes, indicating that alpha-amanitin resistant transcription was occurring. After 15 minutes the incorporation rate levels off, indicating that polymerase 1 molecules have slowed or stopped incorporating additional [ $^{32}\text{P}$ ]-UTP.

Figure 5

Nuclear run-on  $^{32}\text{P}$ -UTP incorporation  
in the presence of alpha-amanitin.





media supplemented with 10% FBS than those prepared from cells grown in media supplemented with 2% FBS. When nuclei were isolated from cells grown in 10% FBS, treated with RNase A, and allowed to transcribe for 10 minutes in the presence of alpha-amanitin, the purified RNA contained between 0.29% to 0.90% of total [ $^{32}$ P]-UTP added ( $6.38 \times 10^5$  to  $1.98 \times 10^6$  dpm). For nuclei from cells grown in 2% FBS, 0.061% to 0.19 % of the [ $^{32}$ P]-UTP added ( $1.32 \times 10^5$  to  $4.18 \times 10^5$  dpm) was incorporated into the purified RNA.

In order to assess the elongation efficiency of pol I transcription in run-on reactions, aliquots of the purified RNA from these reactions was glyoxylated and resolved on 1.0% agarose gels. The gels were stained with ethidium bromide, but upon viewing under uv light, no banding pattern was evident, suggesting low levels of transcription. The gel was then dried and an autoradiogram was made. The resulting autoradiogram (Figure 6) shows a single large band in each lane. The size of the band is from 160-360 bp, which agrees well with other reported pol I run-on transcript sizes of 200-300 bp (Harrington and Chickarishi, 1987).

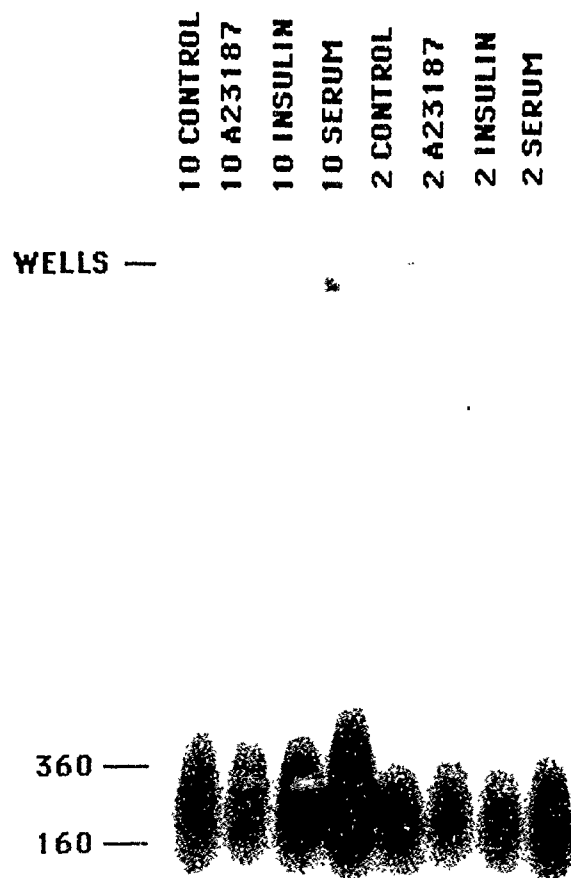
Sollner-Webb and coworkers have estimated that the elongation rate *in vivo* of mammalian pol I is about 30 nucleotides per second (Sollner-Webb and Tower, 1986). Elongation at this rate could yield an 18,000 base long ribosomal RNA after a ten minute reaction. The results obtained in Figure 6 suggest that the *in vitro* run-on reaction is about 2.0% as efficient as the *in vivo* reaction, or that the elongation rate is about 30 bases per minute. This estimated rate is in accordance with observations by other workers (Harrington and Chickaraishi, 1987). It is possible, however, that degradation of the ribosomal RNA has occurred and that the degradation products fortuitously fall into the 300 base size range. One would expect if this were the case that a smear of low molecular weight RNAs would be seen, which is not observed. On the other hand, if no degradation has occurred, one would expect that higher molecular weight RNAs would also be present, representing longer transcripts that were "frozen" as the nuclei were isolated and then labeled on their 3' ends. These transcripts are also not seen. It may be possible that the pretreatment of the isolated nuclei with RNase A degraded transcripts that were previously initiated, and that the run-on transcripts represent only RNA that was transcribed during the run-on reaction.

It is also worth noting the relative intensities of the bands on the

Figure 6. Nuclear run-on rRNA sizes.

Nuclei were isolated from J774.A1 cells that had been grown in either 10% FBS or shifted to 2% FBS for 48 hours and treated with either nothing, A23187, insulin, or serum. Nuclear run-on reactions were conducted in the presence of alpha-amanitin, and total RNA was isolated, dried, and resuspended in 1000  $\mu$ l of DEP-treated water. Twenty  $\mu$ l of each RNA sample were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining and by autoradiography. No bands were visible when the gel was viewed under uv light (not shown). The autoradiograph of the resolved RNA is shown. Size markers (bp) and the wells of the gel are indicated.

FIGURE 6. Autoradiograph of nuclear run-on RNA.



autoradiogram in Figure 6. Cells that were grown in 10% FBS and then were stimulated with either A23187, or additional serum all show increased transcription when compared to the untreated control cells. 10% cells treated with TPA do not show an increase. Likewise, cells that were grown in the presence of 2% FBS showed increased transcriptional activity when stimulated with serum. Finally, the relative amounts of transcription for cells grown in 10% FBS were greater than for cells grown in 2% FBS regardless of stimulating agent.

Run-on transcription was probed by using ribosomal DNA sequences specific for the upstream intergenic spacer region, the enhancer region, the external transcribed region, and the 28S coding region.

We wanted to assess the amount of stimulation of ribosomal RNA transcription that was conferred by treatment with the above reagents. We also wanted to assess if this resulted in increased numbers of active pol I transcription complexes in all regions of the ribosomal gene, or if it was confined to certain regions only. In order to accomplish this, we chose probes that corresponded to the intergenic spacer region (IGS), the enhancer region (ENH), the external transcribed spacer (ETS), and the 28S (28S) coding region (Figure 7).

Most workers agree that the ETS and 28S region are actively and heavily transcribed. There is less consensus for transcription in the IGS and the ENH region. (for example, Kuhn and Grummt, 1987; Wood *et al.*, 1984). Located upstream of the ENH is a formation called the spacer promoter. (Sollner-Webb and Mougey, 1991; Reeder, 1989; Kuhn and Grummt, 1987). The spacer promoter shares a high degree of sequence homology with the gene promoter. (Kuhn and Grummt, 1987). However, it is not clear what function the spacer promoter has *in vivo*.

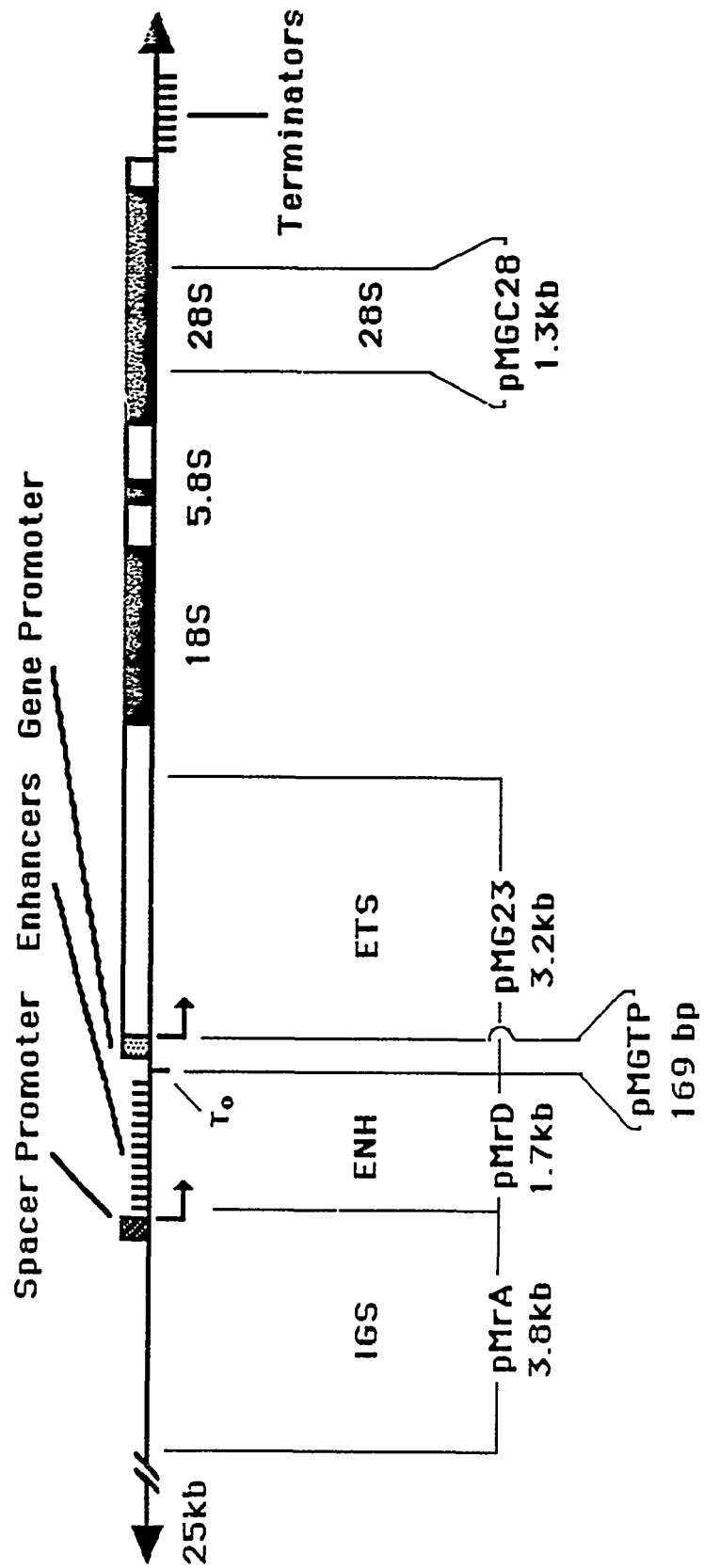
The probe used to detect transcription in the IGS region consists of a 3.8 kb EcoRI-Sal I fragment. This fragment includes at its 3' end the entire spacer promoter. Transcription detection in the ENH region is accomplished by a 1.7 kb Sal I fragment. Transcription detection in the ETS region was accomplished by a 3.2 kb Sal I-Eco RI fragment. This fragment contains the gene promoter located at its 5' end, and the majority of the external transcribed region. It does not contain any of the 18S coding region. Transcription in the coding region is detected by a

Figure 7. Mouse rDNA clones.

Plasmids that contain portions of the rDNA gene are shown. Plasmid pMrA contains 3.8 kb Eco R I-Sal I fragment of the 3' end of the Intergenic Spacer (IGS) region. This fragment contains the spacer promoter region at its 3' end. Plasmid pMrD contains a 1.7 kb Sal I fragment which encompasses the entire enhancer (ENH) region. Plasmid pMGTP contains a 169 bp Sal I-Rsa I fragment that contains sequences immediately downstream of  $T_0$  to the start transcription start site (-168 to +1). Plasmid pMG23 contains a 3.2 kb Sal I fragment which contains most of the external transcribed (ETS) region, starting immediately downstream of  $T_0$  terminator. Plasmid pMGC28 contains a 1.3 kb Sal I-EcoR I insert from the 28S region. Transcription start sites for the spacer promoter and the gene promoter are shown arrows under the respective promoter regions. Terminators are shown by vertical lines descending from the gene. The  $T_0$  terminator is designated by " $T_0$ ."

# Mouse rDNA Clones

Figure 7



1.3 kb Sal I fragment that is located in the 28S coding region, approximately 7 kb downstream from the transcription start site.

Determining the specificity of the probes.

In order to determine the specificity of the various ribosomal DNA probes used, as well as a measure of hybridization efficiency, Southern blot analysis was employed. The ribosomal DNA insert from each probe was liberated from its plasmid carrier by restriction enzyme digestion. Liberated fragments were then resolved on 1.0% agarose gels, and blot transferred to Zeta Probe nylon membranes (Dupont). Additionally, plasmid pMGC was constructed. This plasmid contained a 1.0 kb fragment of Lambda phage DNA, which was determined by computer analysis to have a unique DNA sequence that showed no similarity to the ribosomal DNA probes used. The 1.0 kb fragment was ligated into a pGem3Z vector. This construct allowed the T7 promoter of the pGem3Z vector to transcribe the Lambda phage DNA insert into RNA. The Lambda phage RNA could then be used as a measure of RNA hybridization efficiency between hybridization reactions in subsequent hybridization analysis. In order to physically test the computer prediction that ribosomal RNA would not hybridize to the 1.0 kb Lambda DNA fragment, the Lambda DNA insert from pMGC was also liberated by restriction enzyme digestion and resolved by agarose gel electrophoresis along with the above mentioned probes.

A ten minute run-on reaction utilizing cells grown in the presence of 10% FBS was completed, and RNA was isolated according to a modification of the method of Chomczynski and Sacchi (1987). The dried RNA from the run-on reaction was dissolved in 1ml of water and dpms were counted in a liquid scintillation counter. Three  $\times 10^6$  cpm of purified RNA was then added to the hybridization reaction, and allowed to hybridize overnight at 42°C. After completion of the hybridization reaction, the nylon membranes were removed from the hybridization cocktail and replaced with an identical prehybridized membrane. The original membranes were then washed while the second membrane was allowed to hybridize for 72 hours at 42°C, and both were exposed to X-ray film.

Figure 8 A shows the the probe plasmid digestion products and the sizes of the inserts. Panel B shows the autoradiograph produced from the first membrane

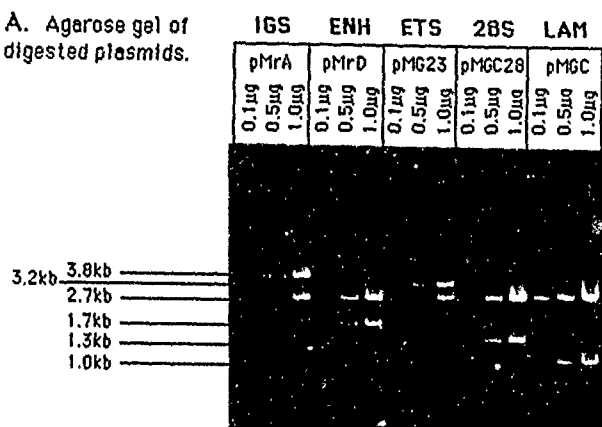
Figure 8. Determining hybridization times and probe specificities.

(A). Plasmids pMrA, pMrD, pMG23, and pMGC28 were digested with restriction enzymes to liberate the inserted rDNA region (pMrA-3.8 kb, pMrD-1.7 kb, pMG23-3.2 kb, pMGC28-1.3 kb). pMGC contains a 1 kb fragment of Lambda DNA, and was used as a non-specific DNA control. pMGC was also digested to liberate the Lambda insert. All plasmids were resolved on a 1% agarose gel, stained with ethidium bromide, and photographed under uv light. The digested vector migrates as a 2.7 kb band. Duplicate gels were made and alkaline transfer to Zeta-probe was accomplished. (B). Ribosomal DNA bound to Zeta-probe nylon membranes was probed with rRNA purified from nuclear run-on assays of J774.A1 cells grown in 10% FBS for 24 hours. Membranes were washed and exposed to X-ray film. (C). An identical membrane was prehybridized and added to the hybridization mixture used to probe 'B.' Hybridization was carried out for 72 hours, after which the membrane was washed and exposed to X-ray film.

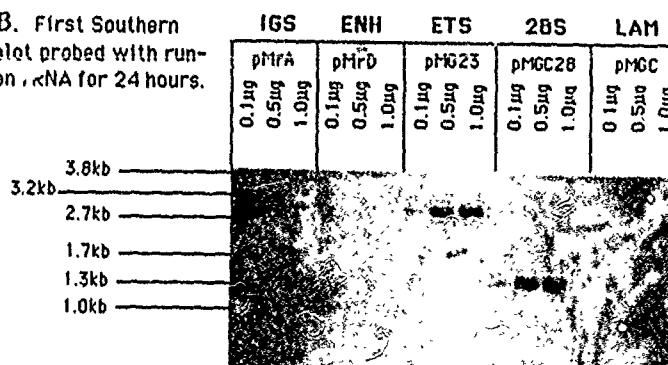


Figure 8

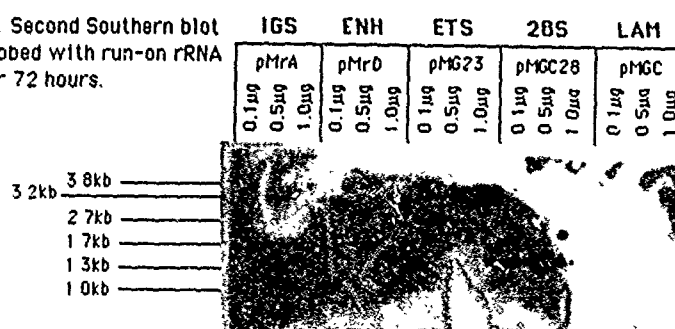
A. Agarose gel of digested plasmids.



B. First Southern blot probed with run-on, rRNA for 24 hours.



C. Second Southern blot probed with run-on rRNA for 72 hours.



hybridized with run-on RNA. Note that there has been no hybridization in either the IGS or ENH probes or to the plasmid vector (pUC9 for pMrA, pMrD, pMG23, and pMGC28, and pGem3Z for pMGC.), but there was significant hybridization to the ETS and 28S regions. We did, however observed significant hybridization to both the IGS and the ENH probes from the second, longer, hybridization (panel C), in addition to more hybridization in the ETS and 28S regions. Also in this second hybridization reaction we noted no significant hybridization to the vector DNA.

The observation that hybridization times on the order of 72 hours were required for detection of transcription in the IGS and the ENH may account for why other workers, using shorter hybridization times, did not see transcription in the IGS or ENH regions (Wood *et al.*, 1984).

From these experiments (Figure 8) we concluded that there was no significant hybridization of run-on RNA to either the plasmid vector, or to the internal control plasmid pMGC. This observation allowed the use of denatured plasmids for dot blot analysis. We also concluded that longer hybridization times (on the order of 72 hours) would increase our ability to detect rare transcripts in the IGS and ENH region.

#### Determination of the amount of probe DNA per dot for dot blot analysis.

If the amount of DNA per dot in a dot blot analysis is not in excess over the amount of RNA available for hybridization to that dot, a quantitation of the amount of RNA present cannot be made. To complicate this matter, we had to be able to resolve transcription signals that would come from heavily transcribed regions of the gene (ETS and 28S) and from regions that produced few, if any, transcripts (IGS and ENH). The problem we faced was that if we diluted the run-on RNA so that we would be in DNA excess on the membrane in a single dot for the heavily transcribed ETS and 28S regions, we could not resolve a signal from the lightly transcribed IGS and ENH regions. We decided to place multiple (6-8) dots of ETS and 28S probe DNA and fewer (2-4) dots for the IGS and ENH probe DNA. We reasoned that the larger number of dots for the ETS and 28S region would serve to hybridize the run-on RNA produced in this region, while allowing a signal to be resolved in the IGS and ENH regions because proportionally more run-on RNA could be loaded into the system.

In order to assess that we were in DNA excess on the membranes to be probed, sets of three identical dot blot membranes were produced utilizing a Bio-Rad Dot Blot manifold. Probe DNA was crosslinked to the membrane in increasing amounts, from 0.5 to 2.5 pico-moles of insert per dot (Figure 9 A). Purified, labeled RNA from a run-on reaction was lyophilized to dryness, and redissolved in 1 ml of DEP-treated water. The dot blot membranes were prehybridized and 1  $\mu$ l, 10  $\mu$ l, or 20  $\mu$ l of run-on RNA was added to the samples. Membranes were hybridized for 72 hours and washed as previously described. Autoradiographs of all three membranes were made on the same Kodak X-OMAT film. The autoradiographs were then scanned using a LKB laser densitometer. The resulting scans were analyzed by comparing the relative area under the absorbance peaks for each probed region of the ribosomal gene.

Figure 9 A shows the resulting autoradiographs for the three separate hybridization reactions. Panel B of Figure 9 shows the relative summed absorbance for each probed region for the three conditions. Panel C of Figure 9 shows the resulting graph, plotting  $\mu$ l of run-on RNA against relative absorbance for each of the probed areas. In an ideal situation, the absorbance for each probed area would increase by a factor of ten between the 1  $\mu$ l and 10  $\mu$ l conditions, and by a factor of two between the 10  $\mu$ l and 20  $\mu$ l conditions. This appears to be the case in both the ETS and 28S regions when comparing the 10  $\mu$ l and 20  $\mu$ l conditions. The 1  $\mu$ l and 10  $\mu$ l conditions do not approach the ideal situation as closely. The differences in absorbance are about 5 fold and 8 fold for the ETS and 28S regions, respectively. These differences may be due to non-linearity in this portion of the curve.

We concluded from these experiments that binding 2.5 pico-moles of probe insert per dot, and using 6-8 dots for the ETS and 28S region would be sufficient to meet the quantitative requirement of membrane DNA excess using 10  $\mu$ l (about 2,000 dpm/ $\mu$ l) of run-on RNA

Figure 9. Determination of DNA excess conditions.

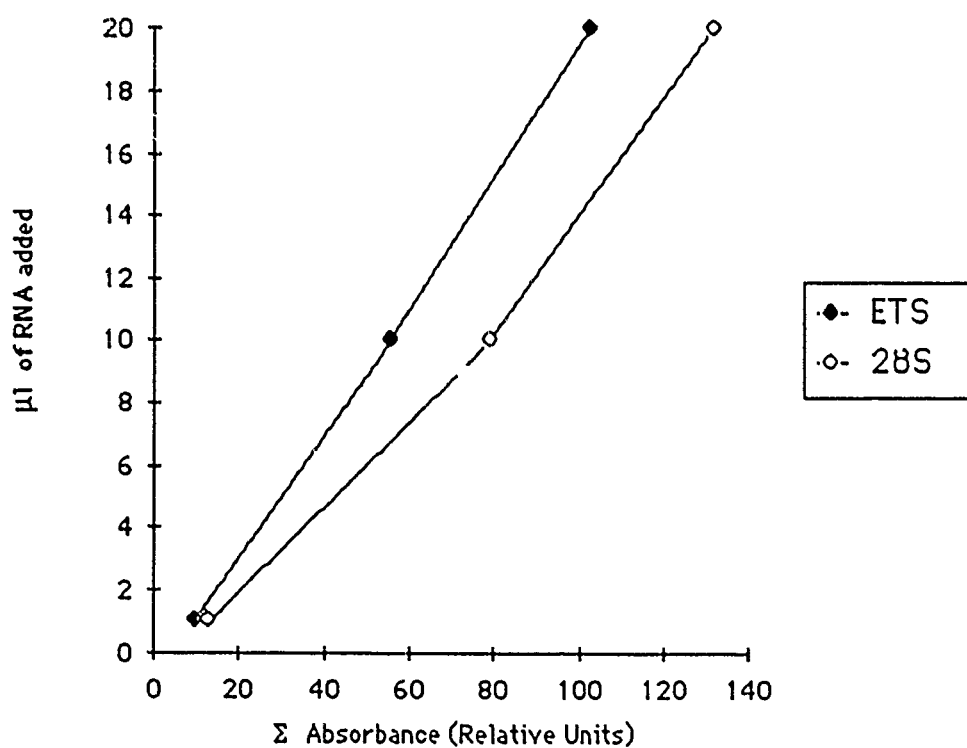
(A). Plasmids containing the IGS, ENH, ETS, and 28S region inserts were denatured under alkaline conditions and dot blotted to Zeta-probe nylon membranes. The dots for the IGS and ENH regions both contained 2.5 pico-moles of insert DNA. The dots for the ETS and 28S region contained varying amounts of DNA, ranging from 0.5 to 2.5 pico-moles of insert DNA. Multiple membranes were constructed. Three identical membranes were probed with rRNA from a nuclear run-on reaction of serum treated 10% FBS cells. The rRNA from the nuclear run-on reaction was isolated, dried, and resuspended in 1000  $\mu$ l of DEP-treated water. Membranes were probed with either 1  $\mu$ l, 10  $\mu$ l, or 20  $\mu$ l of resuspended rRNA. After a 66 hour hybridization the membranes were washed, dried, and exposed to X-ray film. (B). The resulting autoradiographs were scanned with a densitometer, and the area under the resulting peaks was integrated and summed for the individual gene regions for each membrane. The data is presented in relative units. (C). The data in Table B in graph form is for the ETS and the 28S regions.



Figure 9 (continued)

B. Table of  $\Sigma$  Absorbance of DNA Excess Control Experiments.

<u>RNA added</u>	$\Sigma$ Absorbance (Relative Units)			
	<u>IGS</u>	<u>ENH</u>	<u>ETS</u>	<u>28S</u>
1ul	1	2	10	13
10ul	3	3	56	79
20ul	7	6	103	132

C. Graph of  $\Sigma$  absorbance of DNA excess control experiments.

Nuclear run-on reactions from cells grown in the presence of 10% FBS and stimulated with TPA, A23187, insulin, or serum show increased ribosomal RNA transcription.

When nuclei that had been prepared from J774.A1 cells were treated with TPA, A23187, TPA, or FBS, increases in pol I transcription were observed. Cells that were growing in 10% FBS showed larger increases than those that were growing in 2% FBS.

J774.A1 macrophage cells that were grown in the presence of 10% FBS were scraped, pooled, and divided into equal aliquots. Aliquots were then treated with either TPA, A23187, insulin, or additional serum for varying lengths of time. An untreated control aliquot was also processed in the same manner. After completion of treatment, cells were recovered and nuclei were prepared. Nuclei were then frozen at  $-70^{\circ}\text{C}$  where they remain transcriptionally competent for >6 months.

Nuclear run-on reactions were performed either in the presence or absence of alpha-amanitin. RNA was isolated from each sample, and lyophilized to dryness. RNA from each condition was then hybridized to a nylon dot blot membrane containing probe DNA from the IGS, ENH, ETS, and 28S regions of the ribosomal DNA gene. After a 72 hour hybridization the membranes were washed, dried, and autoradiographed. The resulting autoradiographs were then scanned with a LKB laser densitometer to determine the absorbance of each dot. Absorbances were summed for multiple dots, and normalized to the absorbance value of the untreated control condition (control absorbance = 1.0). Bar graphs were then constructed that independently compare the normalized absorbance values of the IGS, ENH, ETS, and 28S regions.

Figure 10 A is a representative autoradiograph of a hybridized membrane. Panel B depicts a representative scan of the autoradiograph in panel A. Panel C shows a bar graph that compares the relative stimulatory effects of the various treatments. The resulting graph is an average of 3-5 hybridizations per point from 3-5 different nuclear preparations in the ETS and 28S regions, and 1-3 hybridizations for the IGS and ENH regions. The reason for the discrepancy in numbers of experiments between the IGS/ENH and ETS/28S regions is because of

Figure 10. Nuclear run-on reactions from cells grown in 10% FBS and treated with TPA, A23187, insulin, or serum.

(A). J774.A1 cells were grown in 10% FBS and treated with either TPA, A23187, insulin, or serum. Nuclei were isolated, and nuclear run-on reactions were performed on equal numbers of nuclei in the presence of alpha-amanitin. Zeta-probe nylon membranes containing 2.5 pico-moles per dot of insert of the IGS (2 dots), ENH, (2 dots), ETS (6 dots), or 28S (6 dots) were probed with the purified RNA. Membranes were washed and exposed to X-ray film. (B). Dot blot autoradiographs were scanned with a densitometer. A representative scan of one of the rows of dots of the 10% FBS control autoradiograph is shown. The IGS and ENH peaks are small, located at 48 mm and 58 mm, respectively. The three ETS peaks are located at approximately 67 mm, 77 mm, and 87 mm. The three 28S peaks are located at approximately 95 mm, 103 mm, and 112 mm. (C). Each treatment condition was scanned by the densitometer. The areas under the curves were summed and averaged for each condition and region of the gene. The control condition average area for each region of the gene was normalized to a value of one. Each treatment condition for that region was then compared to the control value. Values greater than one indicate rRNA transcriptional stimulation. Values less than one indicate a reduction of rRNA transcription. Bar graphs are plotted with S.E. bars for the ETS and 28S regions. The number of samples is designated by 'n.'



Figure 10

A. 10% FBS cells treated with A23187, Insulin, or Serum.

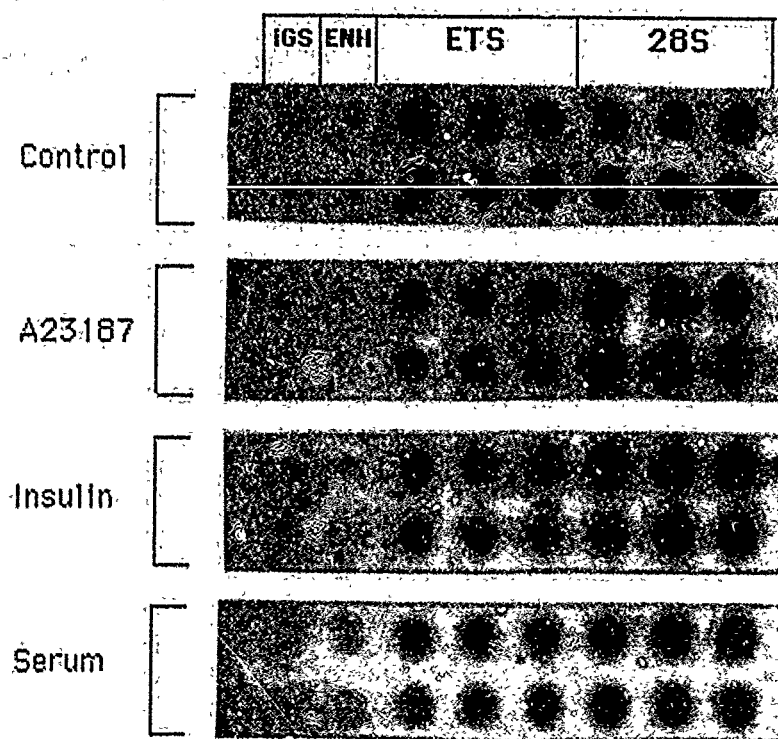


Figure 10 (continued)

B. Representative scan of autoradiograph of 10% FBS cells.

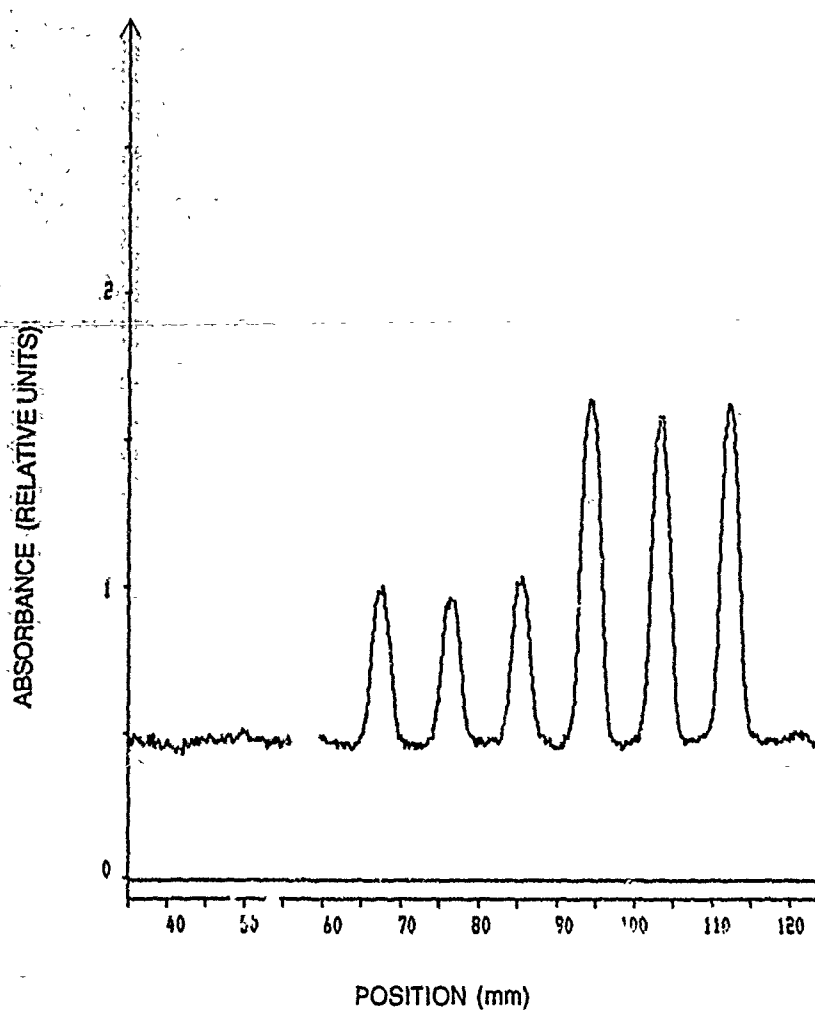
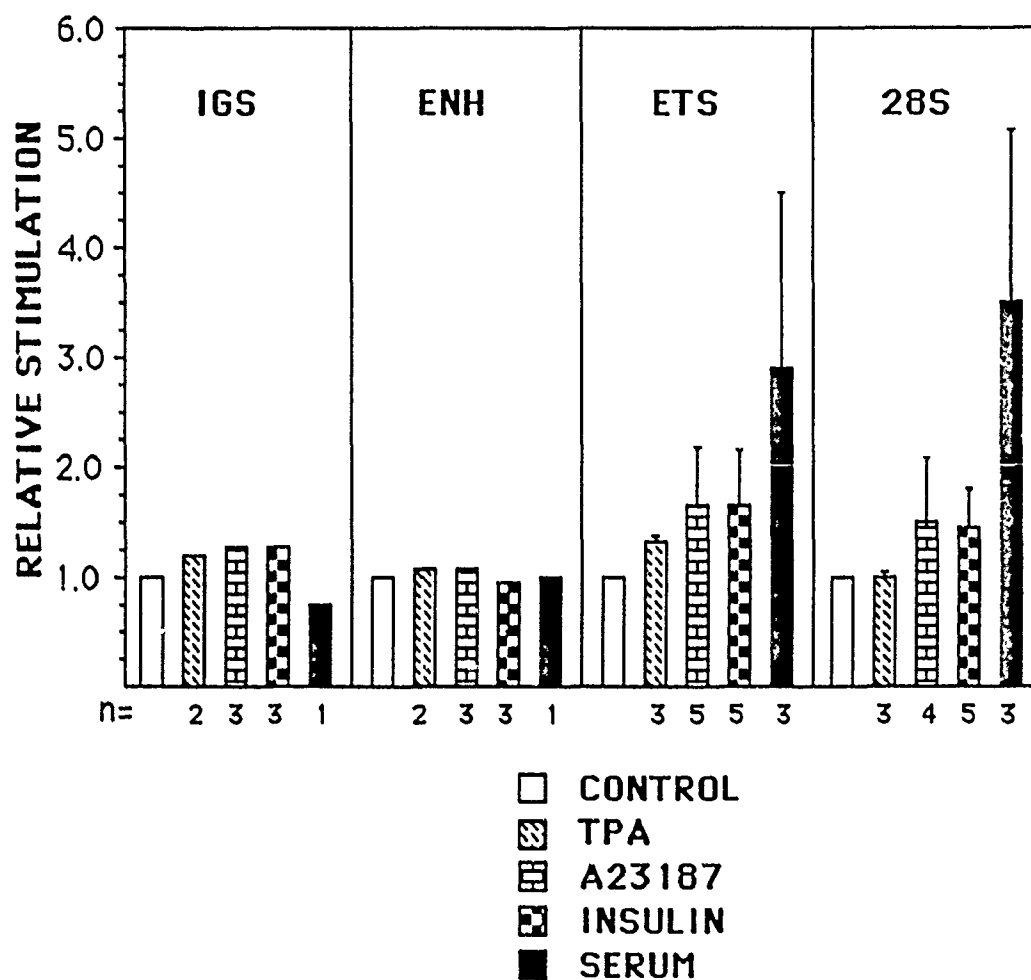


Figure 10 (continued)

C. 10% FBS cells treated with TPA, A23187, insulin, or serum.



the comparatively low levels of transcription that result in the IGS/ENH regions. Not every hybridization resulted in usable data in these regions.

The transcriptional activity of the nuclear preparations varied between preparations. The trend of the data is, however, clear. The IGS region shows modest increases in transcriptional activity for the cells treated with TPA, A23187, and insulin, and, a decrease in transcriptional activity for the serum stimulated cells. These results are consistent with transcriptional activity seen in *Drosophila* SL-2 cell cultures, although not of the same magnitude (Vallett *et al.*, submitted). This result would seem to indicate that transcription is occurring in the IGS region, and that the transcription may be regulated. However, this experiment does not distinguish whether the increase in transcriptional activity is due to the presence of the spacer promoter in the 5' end of the IGS region, or if it is due to increased read-through from the previous upstream sequences.

The ENH region shows very little transcriptional stimulation by any of the stimulating agents used. Since the spacer promoter is located about 70 bp upstream of the 3' end of the ENH region, one could speculate that any transcriptional activity that is initiated from the spacer promoter would be detected downstream in the ENH region. Our data indicates this is not the case.

The ETS region, which includes part of the gene promoter located at the 5' end, showed substantially more transcriptional stimulation than the IGS or ENH regions. TPA stimulated transcriptional activity in this region by about 30%, whereas A23187 and insulin each stimulated transcriptional activity by about 60%. A three hour treatment of the J774.A1 cells with 15% FBS also resulted in a stimulatory effect. The 28S region also showed increased transcriptional activity when treated with TPA, A23187, insulin, or serum. The magnitudes of stimulation were similar to those seen in the ETS region. This region is unique in that it contains a part of the mature 28S ribosomal RNA which is not processed or degraded as are transcripts from the ETS region. It is not yet known how much, if any, of the RNA processing machinery is retained in isolated nuclei, or if it is non-, partial-, or fully-functional if it is retained. However, because this region ultimately becomes part of the mature ribosomal RNA, and is not subject to the degradative processing of the non-coding regions, it may be the best indicator of real transcriptional stimulation. Some workers have suggested that upregulation

of ribosomal transcription occurs coordinately with upregulation of the processing and ribosome assembly apparatus (Vallett *et al.*, submitted, McDermott *et al.*, 1991).

It should be noted that the bar graphs are not directly comparable to one another. This is because each region was normalized to the control condition for that region. As can be seen in panels A and B of Figure 10, the 28S region shows substantially more hybridization than any other region. Because the probes for the IGS, ENH, ETS, and 28S regions are all different sizes and contain different numbers of adenines, direct visual comparisons do not directly reflect hybridization differences. This pattern of hybridization intensities of 28S>ETS>>IGS>ENH is very reproducible, and occurs on the majority of autoradiograms.

From this series of experiments we concluded that rRNA transcription is stimulated when treated with TPA, A23187, insulin, or serum. The apparent density of pol I molecules was much higher in the ETS and 28S regions than in the IGS or the ENH region. Interestingly, IGS region shows a stimulation of rRNA transcription, but the ENH region does not.

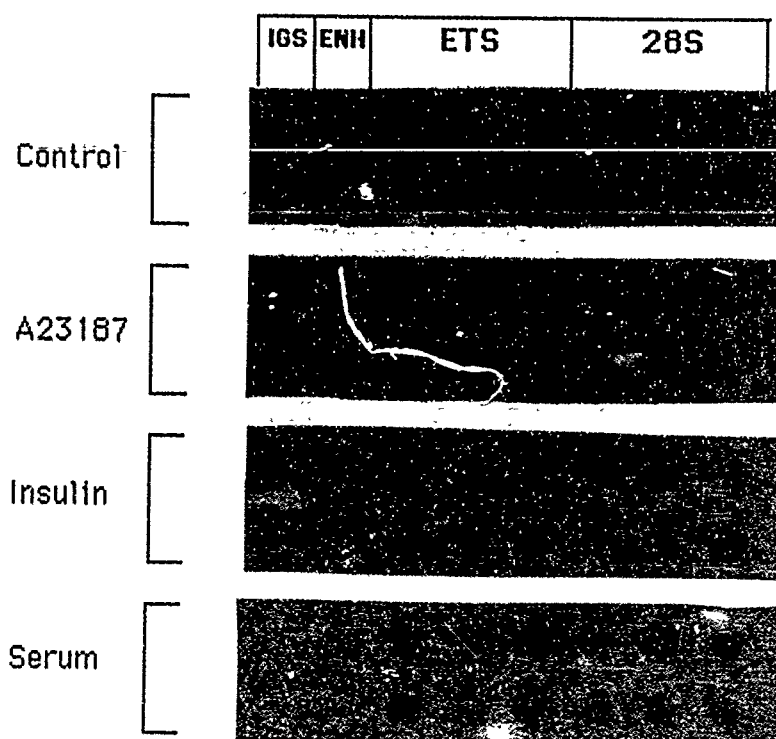
Nuclear run-on reactions from cells shifted to 2% FBS and stimulated with TPA, A23187, insulin, or serum show increased ribosomal RNA transcription.

J774.A1 cells that were shifted to 2% FBS for 48 hours were processed in the same manner as cells grown in 10% FBS. Overall rates of polymerase I synthesis were lower for the nuclear run-on reactions in cells shifted to 2% FBS than for cells constantly grown in 10% FBS. Because of this lower rate of transcription, it was more difficult to detect signals in the IGS and ENH regions, resulting in less usable data for these two regions.

Panel A of Figure 11 shows a representative autoradiograph of a hybridized membrane. Panel B shows a representative scan of the autoradiograph in panel A. Panel C shows a bar graph that compares the relative stimulatory effects of the various treatments. The resulting graph is an average of 3-6 hybridizations per point from 3-5 different nuclei preparations in the ETS and 28S regions, and 1-2 hybridization for the IGS and ENH regions. When 2% FBS cells are treated with TPA, A23187, insulin, or FBS transcription in the ETS and 28S regions is

Figure 11. Nuclear run-on reactions from cells shifted to 2% FBS and treated with TPA, A23187, insulin, or serum.

(A). J774.A1 cells were shifted to 2% FBS for 48 hours and treated with either TPA, A23187, insulin, or serum. Nuclei were isolated, and nuclear run-on reactions were performed on equal numbers of nuclei in the presence of alpha-amanitin. Zeta-probe nylon membranes containing 2.5 pico-moles per dot of insert of the IGS (2 dots), ENH, (2 dots), ETS (6 dots), or 28S (6 dots) were probed with the purified RNA. Membranes were washed and exposed to X-ray film. Note that due to low rates of rRNA transcription, the IGS and ENH show very little hybridization signal. (B). Dot blot autoradiographs were scanned with a densitometer. A representative scan of one of the rows of dots of the 2% FBS control autoradiograph is shown. The IGS and ENH peaks are small, located at 51 mm and 63 mm, respectively. The three ETS peaks are located at approximately 71 mm, 80 mm, and 89 mm. The three 28S peaks are located at approximately 98 mm, 107 mm, and 116 mm. (C). Each treatment condition was scanned by the densitometer. The areas under the curves were summed and averaged for each condition and region of the gene. The control condition average area for each region of the gene was normalized to a value of one. Each treatment condition for that region was then compared to the control value. Values greater than one indicate rRNA transcriptional stimulation. Values less than one indicate a reduction of rRNA transcription. Bar graphs are plotted with S.E. bars for the ETS and 28S regions. A23187, insulin, and serum conditions are lacking from the IGS region and the serum condition is lacking from the ENH region because no usable signal was produced in these regions. The number of samples is designated by 'n.'

**Figure 11****A.** 2% FBS cells treated with A23187, Insulin, or Serum

**Figure 11 (continued)**

**B.** Representative scan of autoradiograph of 2% FBS cells.

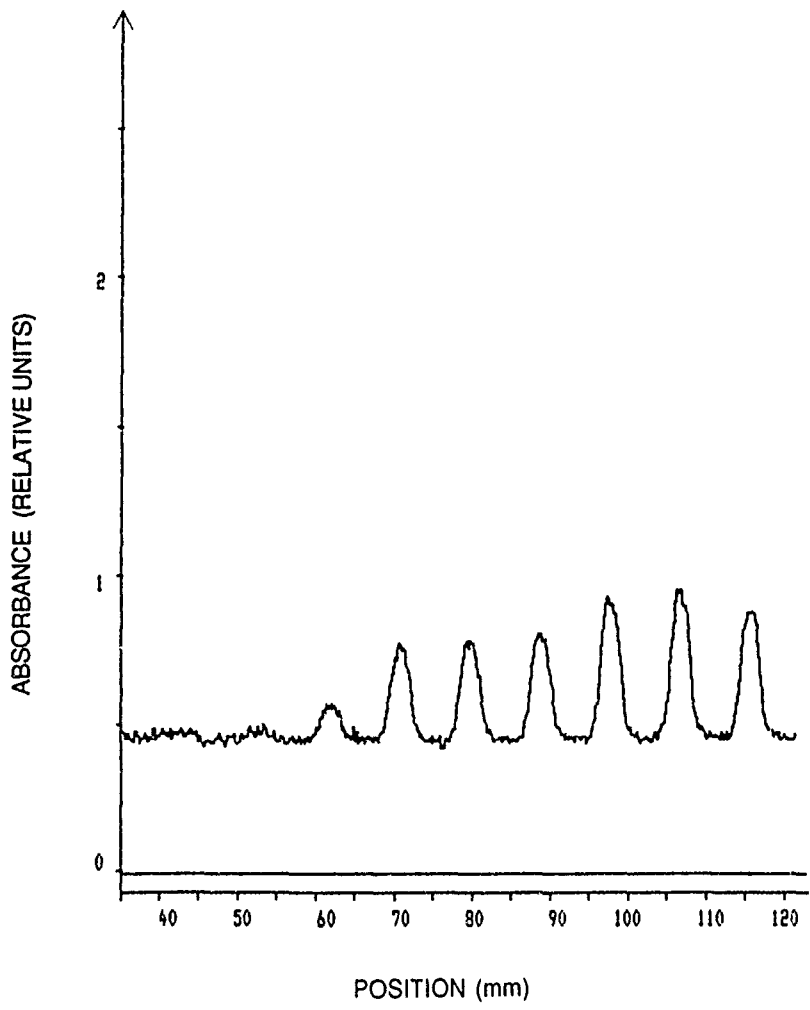
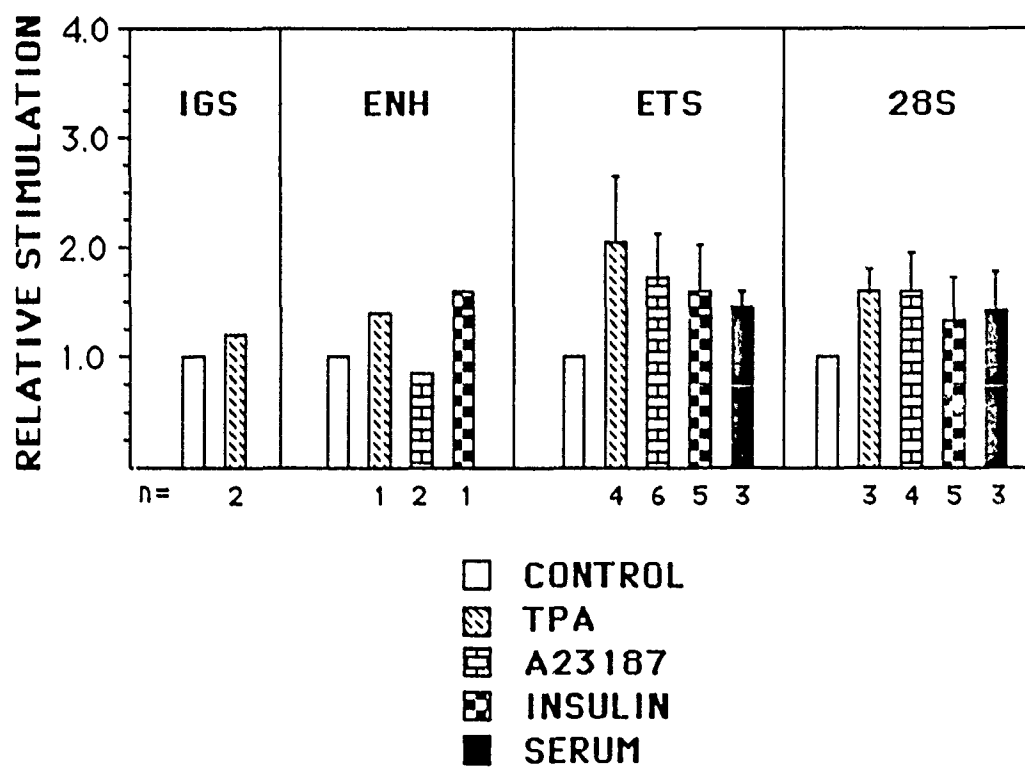




Figure 11 (continued)

C. 2% FBS cells treated with TPA, A23187, insulin, or serum.



stimulated. This is similar to what is seen with 10% FBS cell treated with the same reagents. The general trend of this set of experiments is that although the growth rate of these cell is very slow, they still appear able to respond in some degree to stimulating agents that involve diverse signaling pathways.

Nuclear run-on reactions conducted in the absence of alpha-amanitin produce similar patterns in cells grown in 10% FBS, but much different patterns in cells shifted to 2% FBS.

Cells grown continuously in 10% FBS or shifted to 2% FBS were prepared in the same fashion as described above. However, the nuclear run-on reactions were conducted in the absence of alpha-amanitin. The resulting RNA was harvested and hybridized as previously described.

Panel A of Figure 12 shows a table that compares the results of nuclear run-on experiments normalized to the unstimulated control of the ETS or 28S regions for run-on reactions conducted in the presence or absence of alpha-amanitin. The table compares results from transcriptional stimulation by TPA, A23187, insulin, or serum. Experiments which showed a stimulation of greater than or equal to control are tallied against experiments which showed negative stimulation compared to the control. Cells grown continuously in the presence of 10% FBS show little difference when compared in this manner (Figure 12 B). However, cells that were shifted to 2% FBS for 48 hours show a marked difference in the ability of the stimulating agents to produce a transcriptional upregulation in pol I in the absence of alpha-amanitin (Figure 12 C).

The reason for this discrepancy is not clear. It may be caused by transcripts from both polymerase II and III non-specifically hybridizing to the probe DNA and obscuring the transcriptional increases that have occurred in polymerase I in the run-on experiments conducted without alpha-amanitin, although based on the control data presented in Figure 6 A and other work in this lab (Vallett *et al.*, submitted) this may not be the case.

From these experiments we concluded that alpha-amanitin is an essential ingredient in the run-on reaction. The reason that 2% FBS cells show a marked difference in the ability of stimulating agents to produce a rRNA transcriptional upregulation in the absence of alpha-amanitin is unknown. Because of these

Figure 12. Nuclear run-on reactions conducted in the absence of alpha-amanitin.

(A). Comparison of Nuclear run-on reactions with and without alpha-amanitin. Nuclear run-on reactions were conducted both in the presence and absence of alpha-amanitin for cells grown continually in 10% FBS and cells shifted to 2% FBS for 48 hours. Total RNA was isolated from the nuclear run-on reactions and used to probe identical Zeta-probe membranes that had 2.5 pico-moles of rDNA insert (IGS, 2 dots, ENH, 2 dots, ETS, 6 dots, and 28S, 6 dots) bound per dot. Membranes were washed, and exposed to X-ray film. Resulting autoradiographs were scanned with a densitometer and the area under the resulting peaks was determined. Areas were averaged for the ETS and 28S region, and normalized to the control condition. The table shows the results of these experiments. The number of different experiments that resulted in a rRNA transcriptional stimulation of greater than or equal to the normalized control value (1) and the number of experiments that resulted in a decrease in rRNA transcription relative to the control value (less than one) are shown for experiments conducted in the presence (+) and absence (-) of alpha-amanitin. (B). Bar graph showing the relative rRNA transcriptional stimulation for nuclear run-on reactions of cells grown in 10% FBS and conducted in the absence of alpha-amanitin. The average relative stimulation of each region for each treatment condition is normalized to the control condition (1). The number of samples is shown by 'n.' (C). Bar graph showing the relative rRNA transcriptional stimulation for nuclear run-on reactions of cells shifted to 2% FBS for 48 hours and conducted in the absence of alpha-amanitin. The average relative stimulation of each region for each treatment condition is normalized to the control condition (1). The number of samples is shown by 'n.'

Figure 12

A. Comparison of Nuclear Run-on Reactions  
With and Without Alpha-Amanitin.

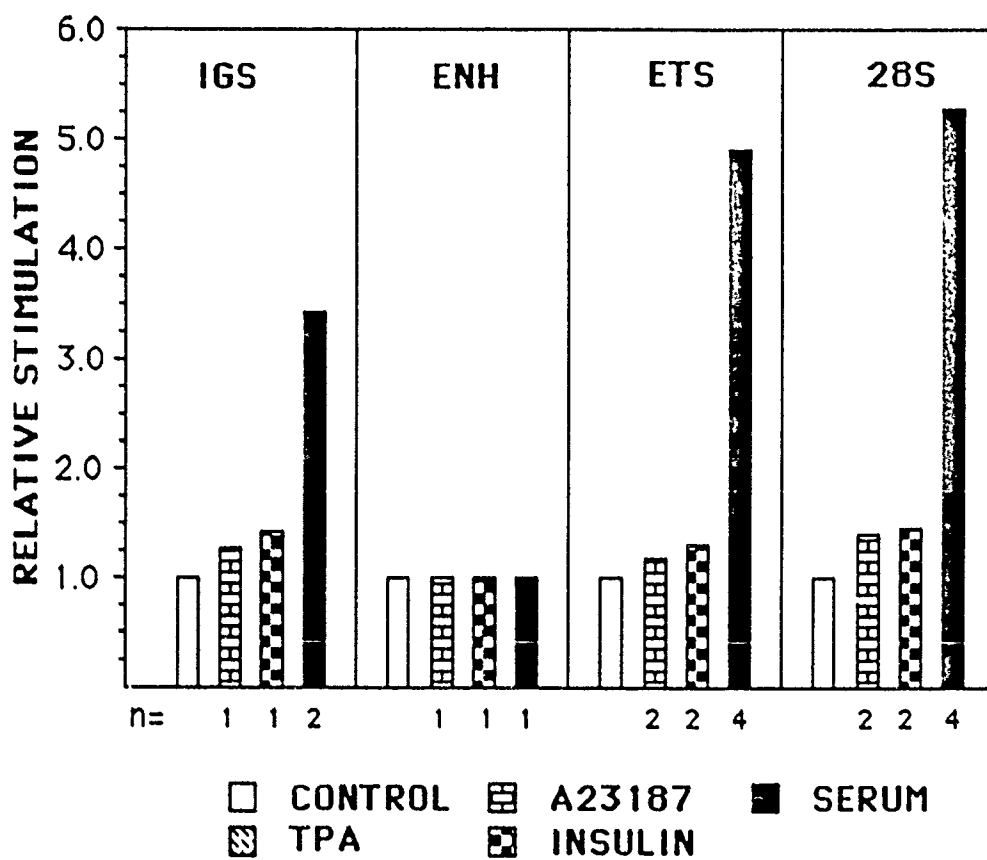
10% Cells ETS Region			10% Cells 28S Region		
	Alpha- Amanitin			Alpha-Amanitin	
	+	-		+	-
TPA $\geq 1$	3	na	TPA $\geq 1$	2	na
< 1	0	na	< 1	1	na
A23187 $\geq 1$	4	1	A23187 $\geq 1$	4	1
< 1	1	1	< 1	1	1
Insulin $\geq 1$	4	2	Insulin $\geq 1$	4	1
< 1	1	0	< 1	1	1
Serum $\geq 1$	3	4	Serum $\geq 1$	3	4
< 1	1	0	< 1	0	0

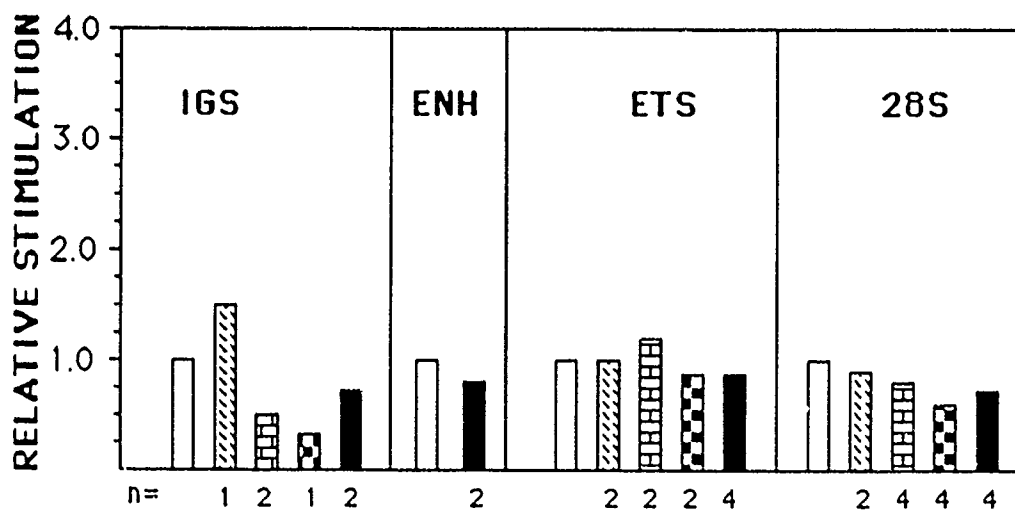
2% Cells ETS Region			2% Cells 28S Region		
	Alpha- Amanitin			Alpha-Amanitin	
	+	-		+	-
TPA $\geq 1$	4	2	TPA $\geq 1$	3	na
< 1	0	0	< 1	1	na
A23187 $\geq 1$	6	1	A23187 $\geq 1$	3	0
< 1	0	3	< 1	3	4
Insulin $\geq 1$	4	0	Insulin $\geq 1$	3	0
< 1	1	4	< 1	2	4
Serum $\geq 1$	3	1	Serum $\geq 1$	3	1
< 1	0	3	< 1	0	3

Figure 12 (continued)

B. Nuclear run-ons without alpha-amanitin of 10% FBS cells.



C. Nuclear run-ons without alpha-amanitin of 2% FBS cells.



Therefore, we limited the data we used to only that which came from run-on experiments in the presence of alpha-amanitin.

1. loading density is different for different parts of the

The apparent pol I loading density is different for different parts of the ribosomal RNA. To compare the apparent polymerase I loading on ENH, ETS, and 28S regions by normalizing the probes for length and uridine content of the RNA transcripts that could hybridize to each probe. Because [ $^{32}\text{P}$ ]-UTP was used as the radioactive label in the nuclear run-on experiments, the autoradiographs reflect the number of RNA incorporated [ $^{32}\text{P}$ ]-UTP molecules that hybridize to each probe. We normalized the adenine content of each DNA probe, and then set the summed absorbance of the ETS region for each treatment condition equal to one. In this manner an estimate of pol I loading across the ENH, ETS, and 28S could be made. These estimates are a "snapshot" of the apparent pol I loading and may not reflect the true situation *in vivo* because they do not take into account the possible effects of ribosomal RNA processing, degradation, or other effects such as polymerase pausing or promoter occlusion.

Panel A of Figure 13 contains a table that presents the ratios of ENH : ETS : 28S for each stimulation condition. Each condition is normalized to ETS = 1.0. Panel B of Figure 13 shows a bar graph of apparent pol I loading with the 28S region compared to the ETS region in cells that were grown continuously in 10% FBS and then processed for nuclear run-on reactions. The ETS is given a normalized value of one, against which each condition of the 28S region is compared. We chose the ETS region as a reference to normalize against because it contained the transcription initiation site. Panel C of Figure 13 depicts a similar bar graph for cells that were shifted to 2% FBS prior to processing for nuclear run-on reactions.

It is interesting to note that the ratios of polymerase I loading between the ETS and the 28S regions are about 1 : 5 for each condition in the 10% cells, and about 1 : 3.3 for every condition in the 2% cells. This result suggests that the stimulatory effects of TPA, A23187, insulin, and additional serum are exerted in a global fashion across the gene. If this were not the case, one might expect to see

Figure 13. Comparison of apparent polymerase I density for different regions of the rDNA gene.

(A). To compare different hybridization signal intensities observed between the ENH, ETS, and 28S regions of the gene, the number of labeled bases that were available to hybridize to the three regions were normalized to the ETS region (ETS = 1). Ratios of ENH : ETS: 28S are shown for nuclear run-on reactions in the presence of alpha-amanitin for cells grown in 10% FBS and cells shifted to 2% FBS for 48 hours. After hybridization, the membranes were treated with 33 mg/ml RNase A for 30 minutes. 'nd', not determined. (B). Bar graph of nuclear run-on reactions conducted in the presence of alpha-amanitin from cells grown in 10%FBS. Bar graph compares normalized ratio of labeled bases available for hybridization in the ETS and 28S region (ETS = 1). Bar graphs shown with S.E. bars. Number of samples indicated by 'n.' Significant difference ( $p=0.05$ ) denoted by '\*.' (C). Bar graph of nuclear run-on reactions conducted in the presence of alpha-amanitin from cells shifted to 2%FBS for 48 hours. Bar graph compares normalized ratio of labeled bases available for hybridization in the ETS and 28S region (ETS = 1). Bar graphs shown with S.E. bars. Number of samples indicated by 'n.' Significant difference ( $p=0.05$ ) denoted by '\*.' (D). Same data as presented in 'A', however, the ENH region is now normalized to one, to better show the differences between the ETS and 28S regions. 'nd', not determined.

Figure 13

A. Table of Ratios of ENH : ETS : 28S

10% CELLS				2% CELLS			
	ENH	ETS	28S		ENH	ETS	28S
Control	0.22	1	5.15	Control	0.30	1	3.77
TPA	0.23	1	4.79	TPA	0.13	1	3.70
A23187	0.14	1	4.84	A23187	0.19	1	3.22
Insulin	0.19	1	5.17	Insulin	0.36	1	3.34
Serum	0.08	1	5.62	Serum	nd	1	3.33

B. Graph of ratios of ETS : 28S for 10% FBS cells.

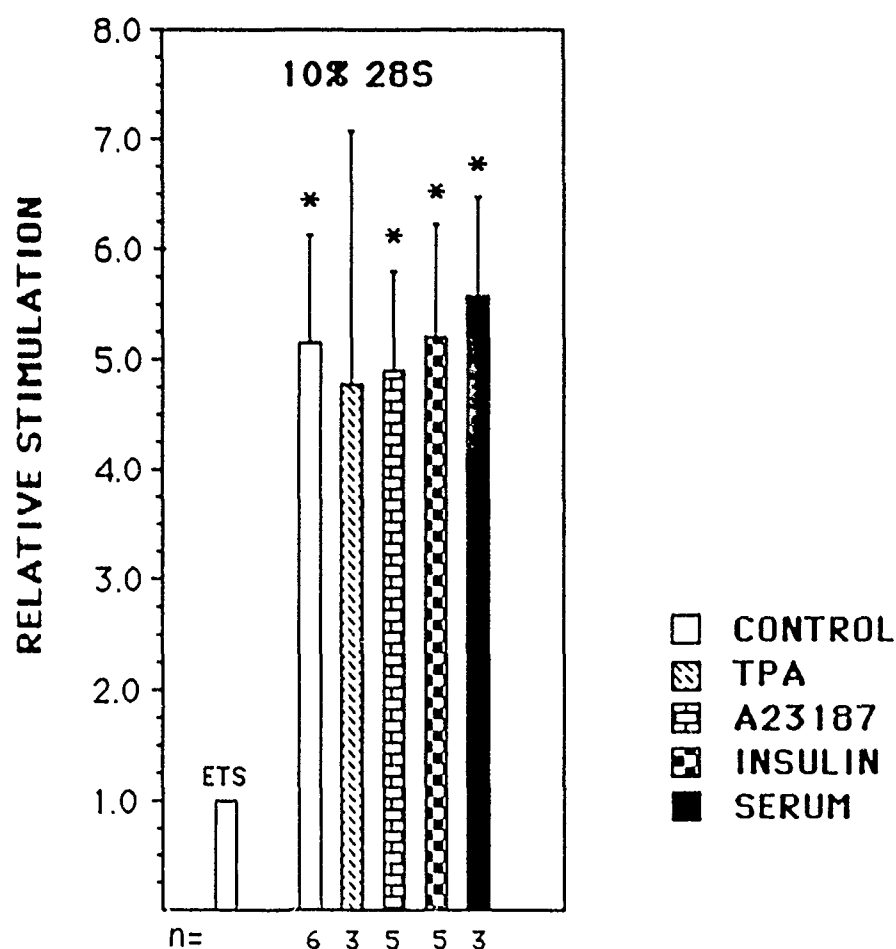
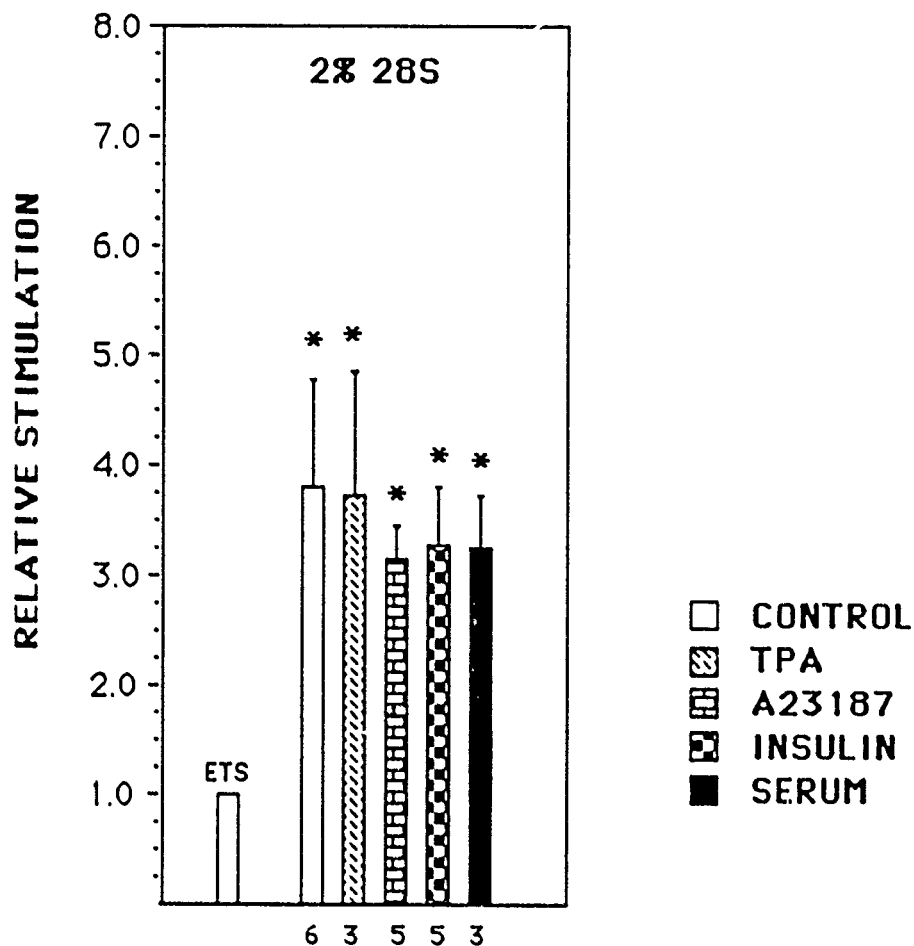




Figure 13 (continued)

C. Comparison of ETS and 28S regions of 2% FBS cells.



D. Table of Ratios of ENH : ETS : 28S

10% CELLS				2% CELLS			
	ENH	ETS	28S		ENH	ETS	28S
Control	1	4.5	23.4	Control	1	3.3	11.4
TPA	1	4.4	20.8	TPA	1	7.7	28.5
A23187	1	7.1	34.6	A23187	1	5.3	16.9
insulin	1	5.3	27.2	Insulin	1	2.8	9.3
Serum	1	12.5	70.3	Serum	nd	nd	nd

changes in the ratios between the ETS and the 28S region. A simple explanation of these results would be that as stimulating agents increase the number of pol I molecules that initiate at the start site, the processing machinery that clips and degrades the ETS portion to the pre-ribosomal RNA transcript is also upregulated in a coordinate manner. This idea is consistent with the data presented in Figure 8 C, which suggested that the ENH region showed little or no changes in transcription when stimulated with TPA, A23187, insulin, or additional serum. The same data presented in panel 13A is presented again in panel 13D. In panel 13D, however, the ETS and 28S regions are now normalized to the ENH region (ENH = 1.0). If the ENH region is indeed insensitive to stimulation, then the data in panel 13D suggests that the increases in transcription are a result of increased initiation from the gene promoter that occurs concurrently with an upregulation of the ribosomal RNA processing machinery that then would keep the ETS : 28S ratios relatively constant.

The difference that is observed between the cells grown continuously in 10% FBS and the cells shifted to 2% FBS may reflect an increase in transcripts from the ETS region relative to the 28S region or a decrease in transcripts in the 28S region relative to the ETS region. The cause of this ratio change is unclear at this time. One hint to the cause of this ratio change may be the absolute lower level of ribosomal RNA transcription that is seen in the cells that were shifted to 2% FBS when compared to the cells grown continuously in the presence of 10% FBS. The cells grown continuously in the presence of 10% FBS were approximately 1.5 to 2.5 (data not shown) times more active in transcribing ribosomal RNA than were the cell that had been shifted to 2% FBS. This overall lower transcriptional activity of the 2% cells may reflect the inability of the cell to operate either the ribosomal RNA processing machinery and/or the transcription machinery in an efficient manner. Inefficient processing could account for a decrease in degradation of the ETS transcripts, thereby tending to lower the ratio of ETS to 28S hybridization. The ratio between the ETS and the 28S would change because there would be relatively more ETS transcripts compared to 28S transcripts. Inefficient transcription may result in pausing of the polymerase I complex in the ETS region, which could result in fewer polymerase I complexes transcribing into the 28S region. This situation would also lower the ratio of ETS to 28S

transcripts, but in this case the ratio would change because of relatively fewer 28S region transcripts compared to the ETS transcripts.

From these experiments we concluded that more transcripts are available for hybridization in the 28S region than in the ETS or the ENH. Cells that were grown in 10% FBS show approximately 5 times as many transcripts available for hybridization in the 28S region as in the ETS region. The ratio of transcripts in the 28S compared to the ETS is about 3 to 1 for cells grown in 2% FBS. The reason for the difference between the 10% and 2% cells is unknown. Finally, the overall level of transcribing pol I molecules is approximately 1.5-2.5 times higher in cells grown in 10% FBS than in cells grown in 2% FBS.

Transcription of the IGS occurs upstream of the IGS spacer promoter region.

Based on the hybridization pattern that was observed in Figure 7 for the IGS region, we wanted to determine if the transcripts that hybridized to the IGS probe, pMrA, were initiated at the spacer promoter or from somewhere further upstream. We digested plasmid pMrA with the restriction enzyme Acc I. This digestion liberated three fragments from the plasmid which were then resolved by electrophoresis on a 1.0% agarose gel. Figure 14 A shows plasmid pMrA and the three AccI sites. The 1.0, 1.2, and 1.6 kb fragments were cut out of the gel, and purified by the GeneClean procedure. The purified fragments were then bound to nylon membranes by dot blotting followed by uv crosslinking and baking in an evacuated environment. The membrane was then probed with ribosomal RNA extracted from a nuclear run-on experiment. Panel B of Figure 14 shows the resulting autoradiogram.

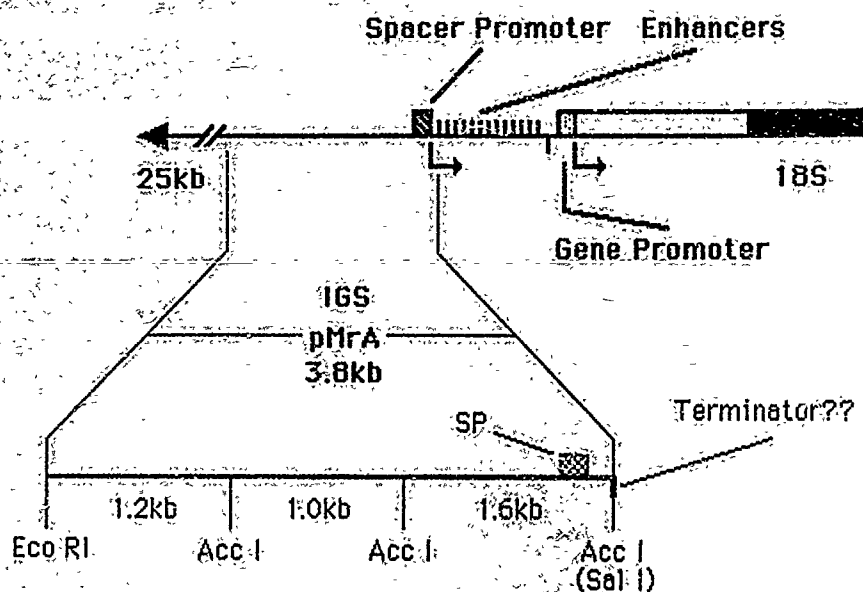
This experiment suggests that transcription is occurring in the IGS region upstream of the spacer promoter. However, the source of this transcription remains unknown. It is unlikely because of the high levels of alpha-amanitin that the transcription is from either polymerase II or III initiating specifically or non-specifically. One possibility is that the transcription may be coming from the upstream ribosomal RNA gene repeat. Another possibility is that the transcription is initiating at some yet undefined promoter element located in the IGS, as the IGS region is poorly characterized and has not been sequenced completely.

Figure 14. Map of IGS region and autoradiogram of IGS transcription.

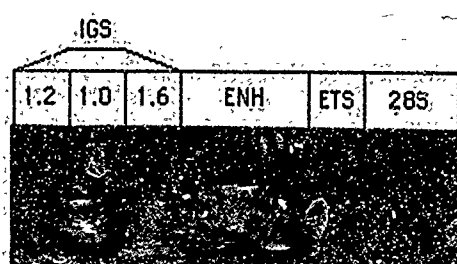
(A). Map showing the 3' 3.8 kb region of the IGS used as a probe and restriction enzyme sites. SP, spacer promoter. Terminator??, putative terminator at Sal I site downstream of spacer promoter. (B) pMrA was digested with Acc I and resolved on an agarose gel. Bands containing the 1.2 kb, 1.0 kb, and 1.6 kb fragments were cut out of the gel, purified, and 1.5  $\mu$ g of each band was crosslinked to Zeta-probe membranes by dot blotting and uv illumination. Membrane was probed with nuclear run-on rRNA from control 10% FBS cells. Autoradiograph is deliberately overexposed to show hybridization occurring in the 1.2 kb, 1.0 kb, and 1.6 kb fragments.

Figure 14

## A. Mouse rDNA IGS region.



## B. Hybridization upstream of spacer promoter.



Because the 1.6 kb fragment of pMrA is the fragment that carries the spacer promoter at its 3' end, we concluded that all of the transcription seen in the ENH region cannot be attributed to initiation at the spacer promoter, and that transcription initiating at some other site is being detected.

Transcription of the 169 bp region upstream of the gene promoter was not detectable using nuclear run-on reactions.

In order to further examine transcription of the spacer promoter, we cloned the 169 bp segment immediately upstream of the gene promoter into plasmid pMGTP. The 169 bp segment of DNA encompasses ribosomal DNA from position -168 to +1 relative to the start site of the primary transcript. The 169 bp fragment contains at its 3' end part of the promoter proximal terminator,  $T_0$ . At the 5' end of the fragment are the gene promoter sequences up to position +1.

We reasoned that if the spacer promoter were initiating transcription *in vivo*, some percentage of the polymerase I molecules would successfully read through the entire enhancer region. Any polymerase I molecules that succeeded in transcribing through the enhancer region might endanger the pre-initiation complex that has formed around the gene promoter by the phenomenon known as promoter occlusion. Since a pre-initiation complex is stable for at least 20 rounds of initiation by polymerase I molecules, it might be advantageous for a cell to terminate transcription from the spacer promoter either in the enhancer or at  $T_0$ . In this way the cell could take advantage of the spacer promoter's ability to attract competent polymerase I molecules to increase the local concentration of polymerase I molecules around the gene promoter, but avoid the problems associated with promoter occlusion. If this is the case, we expected that little or no transcription would be seen in this 169 bp fragment cloned into pMGTP.

Figure 15 A shows the schematic of the local region surrounding the gene promoter. Panel 15 B shows an autoradiograph made of a dot blot containing pMGTP. This autoradiograph is typical of numerous ( $n=40$ ) hybridizations conducted using nuclei prepared from cells that were grown continuously in 10% FBS and either unstimulated or stimulated with A23187, insulin, or additional serum. In no case

Figure 15. Map of gene promoter region and autoradiograms of 169 bp region immediately upstream of the start site.

(A). Map showing 169 bp of the gene promoter region and restriction enzyme sites used to construct pMGTP. Arrows indicate transcription start sites. (B). Two dots each of pMGTP (2.5 pico-moles/dot) were dot blotted and crosslinked to Zeta-probe membranes. Membranes were probed with rRNA purified from nuclear run-ons conducted in the presence of alpha-amanitin from 10% FBS cells that had either been untreated (control) or treated with A23187, insulin, or serum. Membranes were then washed and exposed to X-ray film. Ten repetitions of this experiment were conducted for each condition, and in no case was hybridization detected in the 169 bp region (pMGTP) immediately upstream of the start site. (C). Two dots each of pMGTP (2.5 pico-moles/dot) were dot blotted and crosslinked to Zeta-probe membranes. Membranes were probed with rRNA purified from nuclear run-ons conducted in the presence of alpha-amanitin from 2% FBS cells that had either been untreated (control) or treated with A23187, insulin, or serum. Membranes were then washed and exposed to X-ray film. Ten repetitions of this experiment were conducted for each condition, and in no case was hybridization detected in the 169 bp region (pMGTP) immediately upstream of the start site. TP, plasmid pMGTP.

Figure 15 A.

Mouse rDNA gene promoter region

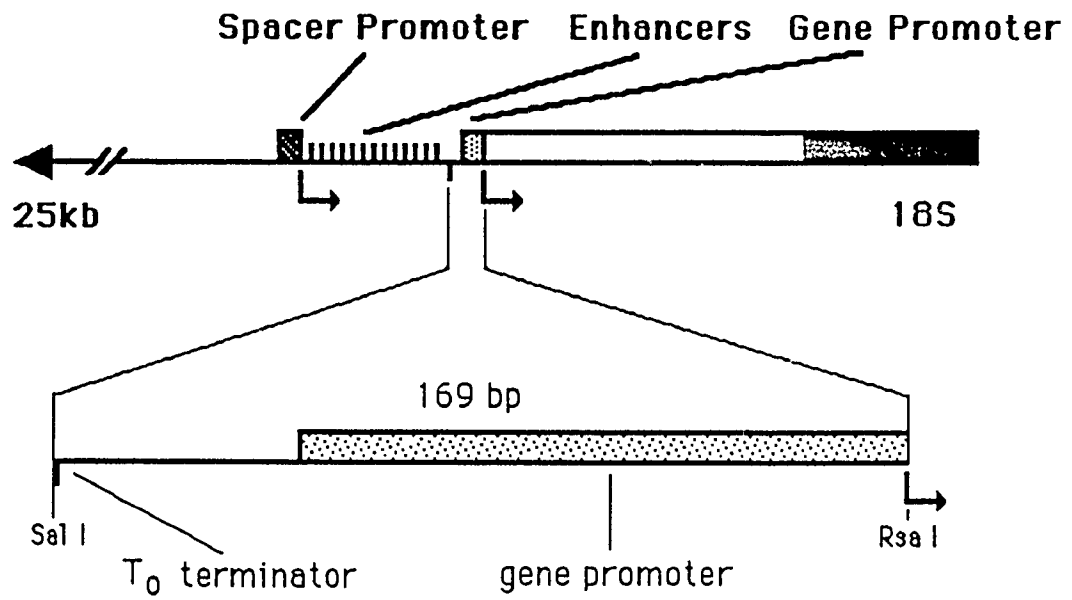
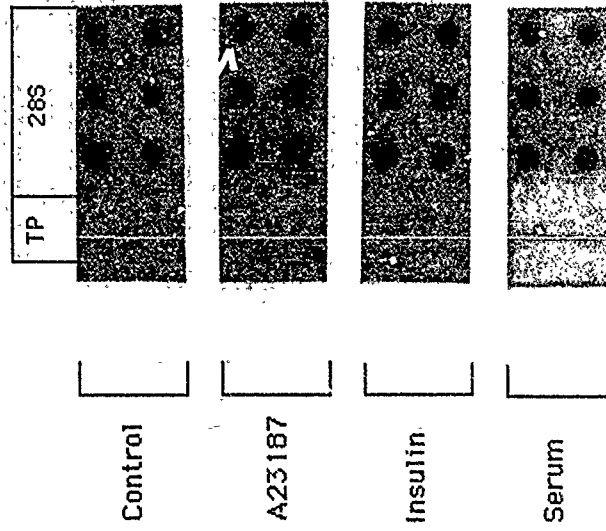


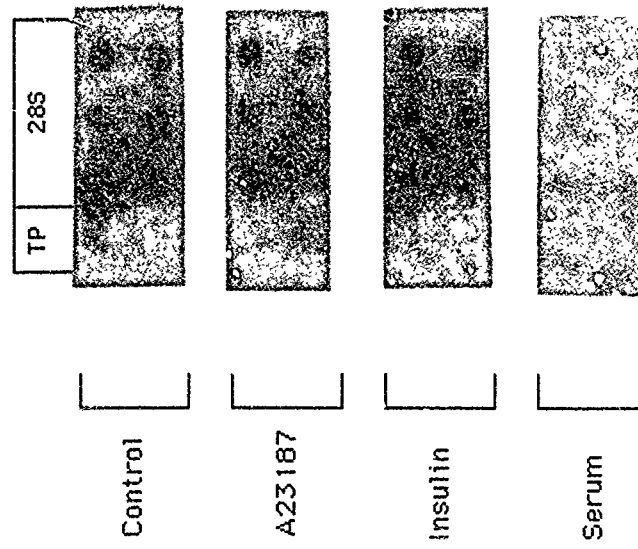


Figure 15 (continued)

C. 2% FBS dot blots of pMGTP and 28S.



B. 10% FBS dot blots of pMGTP and 28S.



was a signal detected in the 169 bp region. Similar experiments were conducted with cells that had been shifted to 2% FBS (Panel 15 C), and again, no signal was detected from the 169 bp region.

We concluded from this experiment that little or no transcription is occurring between the promoter proximal terminator,  $T_O$ , and the transcription start site at +1. This is consistent with the notion that polymerases which initiate at the spacer promoter do not occlude the pre-initiation complex because they are terminated at the promoter proximal terminator,  $T_O$ .

## DISCUSSION

### Control circuits involved in the regulation of ribosomal RNA transcription in J774.A1 mouse macrophage tissue culture cells.

We are interested in understanding the regulatory circuits that control the transcription of rRNA in mouse tissue culture cells. To this end we treated J774.A1 cells with various reagents that are known to be involved in other regulatory pathways and looked for changes in the rate of rRNA transcription. We determined that TPA, A23187, insulin, and serum are all capable of upregulating rRNA transcription in J774.A1 cells.

### J774.A1 cells treated with insulin or serum showed increased incorporation of [<sup>32</sup>P]-orthophosphate into pre-ribosomal RNA.

We treated J774.A1 cells with nano-molar concentrations of insulin or with additional serum and looked for incorporation of [<sup>32</sup>P]-orthophosphate into pre-ribosomal RNA. We observed that both serum and insulin could stimulate the transcription of rRNA, both in cells that had been growing normally and in cells that had been starved for serum. These observations were consistent with similar experiments that had been conducted on both mitotic and non-mitotic *Drosophila* cells in our lab (Vallett *et al.*, submitted; Weber *et al.*, 1991).

The upregulation of rRNA transcription by insulin and serum in systems as disparate as *Drosophila* and *Mus* suggested that similar pathways might be involved in both systems. It also suggested that other reagents, such as TPA and A23187, which had been demonstrated to upregulate rRNA transcription in mitotic and non-mitotic *Drosophila* cells, might also upregulate rRNA transcription in mouse cells (Vallett *et al.*, submitted; Weber *et al.*, 1991).

Nuclear run-on reactions from cells grown in the presence of 10% FBS which had been treated with TPA, A23187, insulin, or additional serum showed increased rRNA transcription.

Based on the observation that insulin and serum stimulated rRNA transcription in both *Drosophila* and mouse cells, we decided to see if mouse J774.A1 cells would also show upregulated rRNA transcription when treated with nano-molar concentrations of TPA or micro-molar concentrations of A23187. We chose nuclear run-ons as our assay because we could use the rRNA produced by the run-on assay to probe representative regions of the rDNA gene. In this manner we could estimate what portions of the rDNA gene were being transcribed at increased rates.

We isolated nuclei from J774.A1 cells which had been growing in media supplemented with 10% FBS after treatment with TPA, A23187, insulin, or serum. Run-on reactions were conducted in the presence of alpha-amanitin to inactivate transcription from pol II and pol III. We used the isolated rRNA to probe dot blots that contained rDNA from the IGS, ENH, ETS, and 28S regions of the gene (see Figure 10).

We observed that TPA, A23187, insulin, and serum all stimulated rRNA transcription. The observation that TPA and A23187 stimulated rRNA transcription was consistent with observations in *Drosophila*, (Vallett *et al.*, submitted; Weber *et al.*, 1991) suggesting that common pathways had been conserved across evolutionary time. The observation that at least four different reagents stimulated rRNA transcription suggests that the regulation of rRNA transcription is under the control of multiple regulatory pathways.

The mode of action of TPA is outlined in Figure 2 A. Since TPA is an analog of DAG, the observation that TPA stimulates rRNA transcription is strong evidence that PKC is involved in the regulation of rRNA transcription. Likewise, the observation that A23187 stimulates rRNA transcription suggests that calcium is also involved in rRNA regulation. The mode of action of insulin is less certain (Figure 2 B). Very little is known about the pathway insulin uses to effect cellular functions. What seems clear is that after insulin binds to its receptor, the receptor tyrosine kinase autophosphorylates and phosphorylates other

undetermined substrates. Somehow this tyrosine kinase influences cellular functions. What can be said about our observation that insulin treatment at physiological concentrations produces a stimulatory effect on rRNA transcription is that we have implicated a peptide hormone in the regulatory control of rRNA transcription.

If the mode of action of insulin is unclear, the mode of action of serum is even less defined. Serum is a complex mixture of polypeptides derived from (in the case of FBS) embryonic tissue. A hallmark of embryonic development is rapid growth which is probably associated with high levels of growth factors, hormones, and other signaling molecules. One would expect that treating cells with serum would be akin to using a shotgun for target practice: *Something* will hit the target. Likewise, *something* in the serum is bound to stimulate cell growth. However, what this something(s) is is not known. The observation that serum stimulates rRNA transcription is, then, somewhat of a fallback position. One does not know what the agents are that are causing the stimulation, but the observation that serum produces the largest stimulation of the reagents tested suggests that multiple pathways are being activated, and can effect rRNA transcription simultaneously.

Nuclear run-on reactions from cells shifted to 2% FBS which had been treated with TPA, A23187, insulin, or additional serum showed increased rRNA transcription.

We observed that J774.A1 cells which had been shifted from 10% FBS to 2% FBS showed a rapid decrease in growth rate (Figure 3). Once shifted to 2% FBS the J774.A1 cells virtually stopped growing within hours of the shift. Doubling times increased from approximately 48 hours to about 14 days. We were interested to see if in this depressed growth state the cells could still respond rapidly to stimulation by TPA, A23187, insulin, or serum. In order to investigate this, we shifted J774.A1 cells to 2% FBS 48 hours before we treated them with either TPA, A23187, insulin, or serum, and then isolated nuclei. We then conducted nuclear run-on reactions in the presence of alpha-amanitin and probed regions of the rDNA gene with the resulting rRNA.

Upon analysis of the resulting autoradiograms, we noted that cells shifted from 10% FBS to 2% FBS could still respond to stimulating agents at about the same levels as cells that had been grown in 10% FBS and not been shifted to 2% FBS before treatment. There was, however, less overall synthesis of rRNA. This result suggests that even at low growth rates the cells possess the necessary factors to utilize the pathways that regulate rRNA transcription but that there is less active transcriptional machinery available to the cell overall.

Serum produced the smallest degree of stimulation in the ETS region in the 2% FBS cell, whereas it produced the largest stimulation in the 10% FBS cells. This interesting observation suggests that serum may be activating multiple pathways effecting rRNA transcription in 10% FBS cells, but fewer pathways in the 2% cells. The reason for this disparity remains unclear.

Stimulation of rRNA transcription is different for different parts of the rDNA gene.

We used rRNA from the nuclear run-on reactions to probe different regions of the rDNA gene in order to gain some idea of changes in the polymerase loading along the gene. Ribosomal RNAs from the nuclear run on reactions were hybridized to four cloned probes (the intergenic spacer region (IGS), the enhancer region (ENH), the external transcribed region (ETS), and the structural rRNA (28S)) (Figure 7).

These experiments suggest that after stimulation different regions of the rDNA gene are differentially loaded with polymerase. The ETS showed the greatest increase in rRNA transcription, followed by the 28S. Both of these regions show similar magnitudes in stimulation (approximately a 1.3 fold increase for TPA, 1.5 fold for A23187 and insulin, and 3.0 fold for serum in 10% cells).

The IGS region is transcribed at a much lower rate than the ETS or 28S regions, showing about 20 fold less hybridization than the 28S region. The surprising result we observed was that the IGS region showed upregulation in response to TPA, A23187, and insulin, but not serum. While the stimulation of rRNA transcription was small (Figure 10 C), it suggests that the IGS is under some form of regulation. The fact that the spacer promoter lies at the 3' end of the IGS probe we used probably is in some way connected with this observation. The

spacer promoter has significant homology with the gene promoter, and may respond to some of the same regulatory signals as the gene promoter.

Another striking observation from these experiments was that transcription of the ENH region does not respond to any of the stimulating agents used. The ENH region contains 13 tandemly arranged repeating elements that have been shown to collectively function as an enhancer element. One of the enhancer's functions is to bind the pol I transcription factor UBF. UBF binding is detected by footprints both on the gene promoter and on the enhancer. The observation that the enhancer is immune to stimulation by TPA, A23187, insulin, or serum may in part be due to a possible pol I terminator located about 70 nt downstream of the spacer promoter at a Sal I site. (I. Grummt, personal communication). If the Sal I site is indeed a terminator, it may be causing pol I molecules which initiated upstream of the spacer promoter, or at the spacer promoter, to terminate transcription and release nascent transcripts. An alternate explanation is that the enhancer may be saturated with bound transcription factors which then impede the progress of any pol I molecule.

Hybridization of RNA from nuclear run-on reactions conducted in the presence of alpha-amanitin are different than those conducted without alpha-amanitin.

We conducted nuclear run-on reactions in the presence and absence of alpha-amanitin. During subsequent hybridizations of nuclear run-on RNA probes to rDNA, we noted distinctly different patterns of hybridization for cells that were shifted to 2% FBS. Interestingly, cells that were grown continuously in 10% FBS did not show significant differences between run-on assays conducted with alpha-amanitin and those conducted without alpha-amanitin.

Nuclear run-on reactions conducted without alpha-amanitin on nuclei derived from 2% FBS cells showed little or no stimulation in both the ETS and the 28S probe regions. Because alpha-amanitin is a potent inhibitor of pol II and III transcription in mammalian cells, we discarded our initial thought that we were seeing non-specific hybridization of transcripts from pol II and III that were masking the changes we expected to see in rRNA hybridization.

Since we did not see any hybridization of rRNA to plasmid vector during Southern analysis, and because we did not see this alpha-amanitin induced disparity in cells that were grown in 10% FBS, we speculate that the lack of stimulation observed is not due to non-specific hybridization from pol II or III transcription.

A rapid rRNA transcription response is seen when treating cells with TPA, A23187, or insulin.

Ribosomal RNA synthesis has long been tied to the growth rate of the cell. Generally, a fast growing cell accumulates rRNA faster than a slow growing cell (for example, McDermott *et al.*, 1991). Similarly, we had previously showed that FBS supplementation correlates with cell doubling time. In order to assess if our treatments by TPA, A23187, or insulin were producing stimulations in rRNA transcription by causing the cell to grow faster, we used short treatment periods. We observed J774.A1 cells which had been shifted to 2% FBS for 48 hours, when treated with 10% FBS, produced increases in rRNA transcription that were not as large as those seen in cells that had been maintained only in 10% FBS (Figure 10 C and 11 C). This led us to believe that the effects of serum starvation were not completely mitigated three hours after refeeding with serum. Because of these observations we reasoned that a shorter treatment period of 15 or 30 minutes might isolate rRNA transcriptional effects due to activation of regulatory pathways from the more global effects of rapid cell growth.

Accordingly, we treated both 10% FBS and 2% FBS cells with TPA for 15 minutes and with A23187 or insulin for 30 minutes and then isolated nuclei for run-on transcription. As has been stated above, both 10% FBS and 2% FBS cells showed rRNA transcriptional stimulation. We attribute this stimulatory effect to activation of regulatory pathways rather than increase in cell growth rate. It remains a formal possibility that the fast temporal effects seen with TPA, A23187, and insulin are simply the first step in a global upregulation of the cell for increased growth rate.



What mechanisms are responsible for the increase in rRNA transcription that occurs when cells are stimulated with TPA, A23187, insulin, or serum?

We have demonstrated that TPA, A23187, insulin, and serum are all capable of upregulating rRNA transcription. TPA stimulation implicates PKC as a factor in gene regulation. A23187 implicates calcium as a factor, and insulin shows that rRNA regulation can be affected by extracellular peptide hormones. The actual mechanisms of the transcriptional stimulation response, as well as the specific pathways that govern it, remain unanswered.

There are several ways that a cell could increase rRNA transcription. One way would be for the cell to transcribe more of the rDNA repeats. Conconi and coworkers (1989) have suggested that rDNA is either bound up into nucleosomes and unavailable for transcription, or free of nucleosomes and actively transcribed. In exponentially growing Friend cells approximately equal amounts of the rDNA are found in each state. It is feasible that freeing up more copies of the rDNA for transcription might be a regulatory method of increasing rRNA transcription. The ultimate example of this would be the amplification of the rDNA in *Xenopus* oocytes during development (Long and Dawid, 1980). An oocyte amplifies its rDNA by about 2,000 fold in order to increase rRNA production.

Another method that a cell could use to increase rRNA transcription would be for the cell to initiate more polymerase at the gene promoter. Transcription of rRNA is preceded by the formation of a pre-initiation complex consisting of UBF and Factor D (Sollner-Webb and Mougey, 1991). Once a stable pre-initiation complex is formed, the polymerase and a tightly associated factor, Factor C, can then bind and initiate transcription. Factor C seems to be subject to phosphorylation by some unknown kinase. When phosphorylated, Factor C, now known as C\*, confers the ability to initiate transcription on the polymerase (Porretta *et al.*, 1991). In the cell at any given time there seems to be two pools of polymerase: bulk polymerase, which cannot initiate transcription, and active polymerase, which can (Tower and Sollner-Webb, 1987). The association of Factor C or C\* with the polymerase seems to be the deciding event in determining a polymerase's ability to initiate (Porretta *et al.*, 1991). Therefore, since Factor C\* seems to be the limiting factor involved in rRNA transcription initiation, the

cell could increase phosphorylation of Factor C to C\*, and concomitantly increase pol I initiation.

There are certainly other models that could be constructed, but the evidence presented by laboratories of Grummt and Sollner-Webb suggest that phosphorylation of Factor C to C\* is an important factor in the regulation of rRNA transcription. The evidence that has been presented in this study supports the hypothesis that phosphorylation is a key element involved in rRNA transcriptional regulation in that it has implicated two known kinases into the regulatory pathway(s). After insulin binds its receptor, the intrinsic tyrosine kinase of the catalytic domain of the receptor is activated. Likewise, the implication of PKC suggests a role for phosphorylation events in the regulation of rRNA transcription. While neither insulin nor PKC are known to act directly on Factor C, it cannot be ruled out that either is acting to phosphorylate other factors which in turn activate the kinase that phosphorylates Factor C.

Nuclei isolated from cells grown in 10% FBS are more transcriptionally active than nuclei isolated from cells shifted to 2% FBS for 48 hours.

We observed by two different measures that nuclei isolated from cells grown in 10% FBS were more transcriptionally active than nuclei isolated from cells shifted to 2% FBS. First, we conducted nuclear run-on reactions and isolated the labeled rRNA. Equal volumes of the labeled rRNA were then resolved on an agarose gel and autoradiographed. As seen in figures 10A and 11A, the rRNA from nuclei isolated from 10% FBS cells produced a larger signal than rRNA from nuclei isolated from cells which had been shifted to 2% FBS.

The second measure we used to determine that 10% FBS cells were more rRNA transcriptionally active than 2% FBS cells was to normalize rDNA probes by taking into account the differences in length and the differences in numbers of labeled nucleotides that would be expected to hybridize to each probe. Even though the 28S probe was shorter (1.3kb) than the ETS probe (3.2kb) and contained a lower percentage of 'T' residues (17.1% T for the 28S, 26.8%T for the ETS), it still showed a stronger hybridization signal than the ETS region (Figure 10 A) indicating that there was apparently more transcription occurring in the 28S region.

When we compared the normalized transcription signals for the ETS and the 28S region, we observed that for cells grown in 10% FBS there was an approximate 1 : 5 ratio regardless of whether the cells had been treated with TPA, A23187, insulin, serum, or not at all. This observation would indicate that there is about five times more transcription occurring in the 28S region than in the ETS region. However, to explain the observation in this fashion, if one assumes that pol I molecules elongate throughout the gene at an equal rate unless sterically hindered, one would have to postulate some form of pol I pausing or stacking up in the structural 18S, 5.8S, and 28S RNA regions that is somehow released during a nuclear run-on reaction. If transcription termination is the slow step in the transcription process, a stacking up or pausing of polymerases that are waiting for termination in the structural rRNA coding portion of the gene might occur. The nuclear run-on reaction presents a "snapshot" of the state of pol I loading at the time of nuclei isolation, and in essence only labels the nascent transcript at its 3' end. If the transcription block is released during a nuclear run-on reaction, a disparity in ETS to 28S signal would be seen.

An alternate explanation for the data would be that processing and degradation of the ETS rRNA is occurring rapidly and in a coordinated manner with the upregulation of the rRNA transcription. Sollner-Webb and coworkers have demonstrated that processing of the 45S pre-rRNA takes place rapidly and that degradation of the clipped off ETS region also occurs rapidly (Craig *et al.*, 1987; Kass *et al.*, 1987). Since processing is known to occur in the nucleolus (Sollner-Webb and Mougey, 1991 and references therein), it seems safe to assume that the processing machinery is isolated along with the nucleus, and retains its activity during the run-on reaction. However, bearing in mind that the nascent transcript of an elongating pol I molecule is processed only at defined points, and that the nuclear run-on reaction's elongation rate is only about 2% as efficient as the *in vivo* condition, it seems unlikely that the 1 : 5 ratio can be solely explained by changes in processing.

We next compared the normalized ETS and 28S regions of cells that had been shifted to 2% FBS before treatment with TPA, A23187, insulin, or serum. Again we observed that the 28S region gave a larger hybridization signal than the ETS region. However, 2% FBS cells showed a reduced ratio of approximately 1 : 3 for the ETS and 28S region, respectively. This reduced ratio might be obtained in at

least two different ways. Either the signal from the ETS region could be larger relative to the signal from the 28S region, or the signal from the 28S region could be smaller relative to the ETS region.

The data does not distinguish between the two possibilities. However, examination of the normalized ratios of the ENH : ETS : 28S, may give a hint as to what is occurring. The ENH region was remarkably immune to treatment with stimulating agents. In 10% FBS cells, if the ENH region is normalized to a value of one, the ETS region shows values for TPA, A23187, and insulin of 4.4 -7.1. A similar range of values is seen when 2% FBS cells are compared in the same way: TPA, A23187, and insulin show ETS values of 2.8-7.7. (Figure 13 D). If, however, the normalized value for the 28S region is compared, one can see that it ranges from 20.8-34.6 for 10% FBS cells and from 9.3-28.5 for 2% FBS cells. An inspection of Figure 13 D will confirm that the 28S values of 2% FBS cells are distinctly lower than the 28S values for 10% cells, while the values for the ETS region are about the same for both the 2% FBS and 10% FBS cells. This suggests that for 2% FBS cells, the 28S region is giving a lower hybridization signal, thereby suggesting that less pol I molecules are transcribing in the 28S region.

An alternate explanation is that processing and degradation of the ETS and ENH transcripts is less efficient in 2% FBS cells, thereby leaving more of these transcripts intact and available to hybridize to the rDNA probes. Regardless of which explanation, if either, proves to be correct, the amount of hybridization signal seen from 10% FBS cells was substantially greater than that seen from 2% FBS cells. From the above observations we concluded that 10% FBS cells are able to synthesize greater amounts of rRNA than 2% FBS cells.

#### The implications of transcription detected upstream of the spacer promoter.

We detected transcription that originated upstream of the spacer promoter (Figure 14 B). The origin of this transcription is unknown at this time. It would seem, however, that fortuitous pol II or III transcription could be ruled out based on the high levels of alpha-amanitin used in the run-on reactions. One possibility is that the transcription detected immediately upstream of the spacer promoter might have initiated at the gene promoter of the previous gene, transcribed the

entire preceding rDNA gene, and not terminated at the T<sub>1</sub>-T<sub>8</sub> terminators, but continued through the entire 25 kb of IGS. The existence of eight terminators in a row at the 3' end of a rDNA gene suggests that termination of a polymerase at that point is an important event. Unterminated polymerase, in addition to causing other unknown problems, certainly would waste cellular supplies of rNTPs in elongating an additional 25 kb to no avail. But, since the DNA sequence of the IGS is mostly unknown, there is no reason to believe that there are not additional transcription initiation points located in the IGS that have not been characterized.

#### Some speculations on the function of the spacer promoter.

The spacer promoter has been conserved across evolutionary time from yeast to human. Still, the function of the spacer promoter is not known. Several models have been put forward to explain spacer promoter function, most notable among these being Moss's read-through enhancement model and Reeder's snowplow model. Moss proposed that pol I molecules bind to the spacer promoter and are then somehow handed off to the gene promoter, thereby assuring a constant supply of initiation competent polymerases. Reeder has proposed that pol I molecules bind to the spacer promoter and then transcribe through the enhancer region towards the gene promoter. In the process of transcribing through the enhancer region, transcription factors such as UBF are plowed off of the enhancer and made available to gene promoters in *cis* or *trans*. Evidence from Sollner-Webb and Paule seem to support Reeder's snowplow model (Henderson *et al.*, 1989; Bateman and Paule, 1988). Their data indicates that a pol I molecule has the ability to displace bound Factor D as it moves across a gene promoter.

The ability to dislodge transcription factors is a two edged sword. While it may be beneficial to free up bound transcription factors from the enhancer region, it would probably be detrimental to rRNA transcription if pol I molecules that initiated upstream of the gene promoter were allowed to transcribe across and disrupt the pre-initiation complex formed on the gene promoter. Since a pre-initiation complex may be active for approximately 20 rounds of initiation, the cell would stand to lose a lot of rRNA transcription if it let pre-initiation complexes be disrupted by upstream polymerases as soon as the complexes were formed. To this end, the function of the promoter proximal terminator, T<sub>0</sub>, causes

polymerase that initiated upstream of the gene promoter to terminate their transcription and release from the template before they occlude the gene promoter.

Moss's read through enhancement model predicts that pol I molecules which initiated at the spacer promoter read through the enhancer region pushing pol I polymerase that have bound to the enhancer but have not initiated, toward the gene promoter until they all terminate at  $T_0$ . Reeder's model also suggests that pol I molecules initiated upstream will terminate at  $T_0$ . Both of these models can be tested by probing for transcription in the region between  $T_0$  and the gene promoter start site. We subcloned this 169 bp region, termed pMGTP, and probed it with nuclear run-on rRNA from 10% FBS and 2% FBS cells that had been treated with A23187, insulin, serum, or nothing. After multiple hybridization reactions (10 separate hybridizations for each condition), we were unable to detect any signal that hybridized to the pMGTP clone. In every case, however, we detected hybridization to the 28S region. We concluded from this experiment that transcription in this region is either very infrequent or nonexistent. In this respect, the predictions of the models of both Moss and Reeder are borne out.

Our data suggests that transcription from the spacer promoter is regulated by the cell. When we treated cells with TPA, A23187, insulin, or serum, we saw increased hybridization to the IGS probe, the probe that contained the spacer promoter. However, the spacer promoter is located close to the 3' end of the IGS clone (about 70 bp upstream from the Sal I site) (Kuhn and Grummt, 1987). One might expect that the majority of rRNA derived from the spacer promoter would hybridize to our ENH clone, since it is immediately downstream of the spacer promoter. However, this does not appear to be the case. The ENH region shows little change in hybridization signal, regardless of treatment with stimulating agents. How is this observation explained, and can either Moss's or Reeder's models account for this observation?

We think that Moss's model would predict that more pol I molecules would initiate at the spacer promoter after the cells were treated with stimulating agents. This could account for the transcriptional stimulation observed in the spacer promoter. However, Moss's model would also predict that more transcription would be seen in the enhancer region, because there would be more 'pusher polymerase' (those that are transcribing) to force uninitiated polymerase

toward the gene promoter. This prediction is not consistent with the observations in the ENH region that we have made.

Reeder's model can accommodate spacer promoter upregulation better than Moss's model. We think Reeder's model would predict more initiation from the spacer promoter following treatment with stimulating agents. However, Reeder's model is somewhat better able to account for the lack of response in the enhancer region if one supposes that plowing off transcription factors is not a trivial task for a polymerase. If polymerase can only dislodge transcription factors with difficulty, and if they are stalled or sometimes dislodged in the process, then this might account for the lack of stimulation seen in the enhancer region.

The most satisfying explanation for this observation is that the putative terminator (I. Grummt, personal communication) located at the Sal I site 70 nucleotides downstream of the spacer promoter is causing newly initiated spacer promoter pol I molecules to terminate and disassociate from the template. However, the putative terminator would have to be relatively weak, in that we have still detected transcription in the enhancer region, indicating that polymerase molecules are passing through the putative terminator and transcribing into the enhancer region.

The data taken as a whole suggests that the cell has maintained a balance between several factors: the strength of the spacer promoter, the strength of the putative terminator, the strength of the  $T_0$  terminator, the distance between  $T_0$  and the gene promoter, the number of enhancer repeated elements, and the strength of transcription factor binding to the enhancer elements. In order for the cell to free up transcription factors that have bound to the enhancer, the spacer promoter initiates a low level of transcription. In order to keep the initiation rate low, the spacer promoter shares limited homology with the gene promoter. Too much homology may result in the spacer promoter's initiation efficiency approaching the gene promoter's efficiency. Too little homology and not enough initiation takes place. The terminators, both the putative spacer promoter terminator and the  $T_0$  terminator are efficient enough to prevent the majority of pol I molecules from occluding the gene promoter, but the putative spacer promoter terminator is inefficient to the degree that some pol I molecules pass into the enhancer region to dislodge transcription factors. Our data would suggest that the putative spacer promoter terminator would act as a brake when rRNA

transcription is upregulated. In this fashion, even though the spacer promoter might be initiating at a higher rate, the putative spacer promoter terminator would slow this rate, letting only the optimal number of pol I molecules through to accomplish transcription factor dislodgment.

The distances between the  $T_0$  terminator and the gene promoter may reflect compromises in efficiency and safety. If the terminator is too far from the gene promoter, transcription-competent polymerase may not be available for immediate reinitiation on the gene promoter. If the terminator is too close, steric interactions between the upstream control element factors and the termination factors may come into play. Finally, the repeated elements in the enhancer are probably optimized to bind transcription factors such as UBF tightly enough so as to act as a 'sink' for these factors, but not so tightly that they cannot be dislodged by an elongating polymerase. The number of repeats is possibly optimized to reflect the same type of compromise: too few repeats will not bind enough transcription factors, whereas too many repeats may sequester too many of the transcription factors.

We have probed individual regions of the rRNA gene for transcriptional activity. We determined that the IGS, ETS, and 28S regions of the rRNA gene are actively transcribed and under regulatory control. The ENH region of the rRNA gene is transcribed at a low rate, which did not change when cells were stimulated with TPA, A23187, Insulin, or serum. We also observed that more transcripts are available for hybridization from the 28S region of the rRNA gene than from the ETS region. This observation suggests that processing of rRNA transcripts from the ETS region occurs rapidly in intact cells. By treating cells with TPA, A23187, insulin, or serum we implicated PKC, calcium, and the insulin receptor in regulatory control pathways of rRNA transcription. Additionally, we demonstrated that transcription is occurring in the IGS upstream of the spacer promoter, but that no transcription was occurring between the  $T_0$  terminator and the +1 start site.



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